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A COMPARATIVE STUDY OF THE ALTERATIONS PRODUCED IN THE PLASMA FIBRINOLYTIC SYSTEM OF MAN BY SURGERY IN THE PRESENCE AND ABSENCE OF INTRAMUSCULAR TRYPSIN[a,b]

E. C. ANDREWS[c], M. MASON GUEST, AND D. R. CELANDER

The clinical use of intramuscular trypsin as a general antiphlogistic agent has become widespread in recent years. Although its beneficial effects have been questioned (Hardy, *et al.*, 1955), reports in current journals indicate that clinical remissions are regularly obtained (Innerfield, 1954; Hopen, 1954; Kryle, *et al.*, 1957; Moser, 1957). The mechanism through which the antiphlogistic effects of small doses of intramuscular trypsin are mediated has not yet been clearly delineated although it has been the subject of much speculation (Martin, *et al.*, 1955; Martin, 1957; Innerfield, 1957; Moser, 1957). Trypsin, when administered intravenously in relatively large doses, apparently acts both directly as a proteolytic agent and indirectly as an activator of the plasma fibrinolytic system (Innerfield, *et al.*, 1952) to bring about reduction in circulating plasma proteins. This communication presents the results of a battery of tests designed collectively to detect any alterations in the plasma fibrinolytic system and associated factors following the administration of intramuscular trypsin.

Methods

Peripheral blood studies were made on 25 patients of the Neurosurgical Service in the John Sealy Hospital at The University of Texas Medical Branch in Galveston. Trypsin was administered intramuscularly to 15 patients immediately after surgery and to 5 patients who did not undergo surgery in doses of 5 mg. of crystalline trypsin in Sesame oil or gelatin every six hours for a period of three days. The remaining 5 patients underwent major surgical procedures without the administration of trypsin. At least two venous blood samples were collected from each patient prior to surgery and/or administration of trypsin. In most cases, at least five postoperative and/or post-trypsin samples were obtained. In patients receiving trypsin, the first sample was collected 6 hours after the first administration of trypsin, and at 24 hour intervals thereafter until 48 hours after discontinuation of trypsin therapy.

All blood drawn except the portion used in the determination of clotting time by the method of Lee and White (1913) was immediately mixed with

4 per cent sodium citrate (9 volumes of blood to 1 volume of citrate). Hematocrit and erythrocyte sedimentation rates were determined immediately after the blood was drawn according to the method of Westergren (1926). Complement was determined quantitatively by the method outlined by Kabat and Mayer (1948) using serum obtained by centrifugation of the Lee-White clots. The serum was stored in sealed ampoules under nitrogen at —70° C. in a dry ice chest until a convenient time for analysis. The C reactive protein determinations were performed as directed by Anderson and McCarty (1950) on serum derived from the Lee-White clots and stored in a conventional deep freeze at —20° C. Fibrinogen was determined essentially by the method of Ware, *et al.* (1947) within three hours after the blood sample was taken. The 24-hour fibrinogen or latent coagulable protein test (described below) was carried out concurrently with the fibrinogen determinations. The remainder of the tests were run on plasma thawed after having been frozen for from two to four weeks. Prothrombin was determined by the Ware and Seegers modification (1949) of the two-stage procedure for prothrombin originally described by Warner, *et al.* (1936). Antifibrinolysin determinations were made by the method of Guest, *et al.* (1947). Prokinase was estimated by the method of Guest and Celander (1956). Spontaneous globulin lytic activity was determined as described below.

Latent coagulable protein test. One ml. of plasma was placed in each of two 50 ml. plastic centrifuge tubes containing 30 ml. saline. One ml. of M/2 phosphate buffer, pH 7.2, 3 drops of 20 per cent calcium carbonate suspension, 0.5 ml. of 1 per cent calcium chloride, and 1 ml. of thrombin reagent, were added rapidly in this order to each tube. At the end of one hour the contents of one of the tubes was centrifuged at 20,000 x *g* for 15 minutes. The pellet of coagulum sedimented by the calcium carbonate was retained and transferred to a 12 ml. graduated conical centrifuge tube, covered with 3 to 5 ml. of saline and washed by kneading with a glass rod. The saline was replaced 3 times. After decanting the washings, the pellet was covered with 0.5 ml. 10 per cent NaOH and hydrolyzed in a boiling water bath for 30 minutes. The remainder of the procedure was identical to that employed in the analysis of plasma fibrinogen. The remaining tube was incubated at 28° C. for 24 hours and treated in a manner identical to that described for the first.

Spontaneous globulin lytic activity. The euglobulin fraction precipitated from 1.0 ml. of human plasma or serum as described by Mellanby (1909) was suspended in 15 ml. of saline and homogenized in an all glass Potter-Elvehjem homogenizer for one minute. Seven ml. aliquots of the resulting suspension were pipetted into each of two 50 ml. plastic centrifuge tubes containing 15 ml. saline. The following reagents were added in order: 1 ml. M/2 phosphate buffer, pH 7.2; 3 drops 20 per cent suspension of calcium carbonate, 1 ml. 0.2 per cent bovine fibrinogen, 0.5 ml. of 1 per cent calcium chloride, and 1 ml. thrombin reagent. At the end of one hour and 24 hours the tubes were processed by the same procedure as used in the determination of latent coagulable protein.

Materials

Trypsin for use in the clinical studies was kindly supplied by the National Drug Co. through the courtesy of Dr. William Swain.

Reagents for the fibrinogen, C reactive protein, and complement determinations were obtained from commercial sources. Bovine fibrinolysin for use in the antifibrinolysin assay and Topical Thrombin were kindly supplied to us by E. C. Loomis of Parke, Davis & Co. Varidase, used as a source of streptokinase in the prokinase determination, was obtained from Lederle Laboratories Division of American Cyanamid through the cooperation of Dr. E. G. Ruegsegger. Bovine fibrinogen was prepared in this laboratory by a slight modification of the method described by Ware, *et al.* (1947).

The 20 per cent calcium carbonate suspension was prepared by reacting solutions of $CaCl_2$ with Na_2CO_3. The resulting precipitate was suspended in 40 times its volume of distilled water, thoroughly mixed and centrifuged (1500 x *g* for 5 minutes). The precipitate was resuspended in a similar volume of distilled water and washed. The washing procedure was repeated three times. The calcium carbonate precipitate was then mixed with distilled water to give a final concentration of 20 per cent by volume after being allowed to settle for 2 hours at room temperature.

Thrombin reagent was prepared by adding the contents of one vial of Parke, Davis Topical Thrombin (5000 NIH units) to 25 ml. physiological saline. The mixture was stirred to dissolve the thrombin and brought to a volume of 50 ml. by the addition of glycerol.

The phosphate buffers of desired ionic strength and pH were prepared from M/2 stock solutions of Na_2HPO_4 and KH_2PO_4. Imidazole buffer, pH 7.25, was prepared as directed by Guest, *et al.* (1948).

Results

The results of the peripheral blood studies are presented in table 1 in terms of average per cent change of post-operative and/

TABLE 1

Effect of Trypsin and Surgery on Various Activities and Components of Peripheral Blood

Determination	Surgery + Trypsin		Trypsin Alone		Surgery Alone	
	Number of Patients	Per Cent Change	Number of Patients	Per Cent Change	Number of Patients	Per Cent Change
Erythrocyte sedimentation rate	14	171	5	47	5	241
Fibrinogen	15	33	5	14	5	14
Latent coagulable protein	14	93	5	—1	5	16
Prokinase	15	— 4	5	2	5	— 12
Antifibrinolysin	15	— 2	5	1	4	— 2
Prothrombin	15	— 24	5	—6	5	— 15
C reactive protein	11	155	5	110	4	266
Complement	11	29	4	23	5	1

or post-trypsin values from control values. While most of the percentage changes are greater than would normally be encountered, the differences between groups, because of the variability among the patients and the relatively small sampling, can be considered at most as trends.

Changes of significant nature were not observed in the whole blood clotting times.

The erythrocyte sedimentation rate (ESR) was most markedly elevated in surgery patients. It is of interest that though trypsin without surgery produced some elevation in the ESR, it did not augment the ESR increase accompanying surgery.

Sixteen of the 20 patients receiving trypsin showed a steady rise in fibrinogen. In 4 of the 16, the increase was preceded by a definite decrease which occurred during the first 6 to 30 hours of trypsin therapy. Rises in fibrinogen were seen in 4 of the 5 patients receiving surgery alone. Surgery plus trypsin seemed to have a somewhat more elevating effect on fibrinogen than did either alone. The number of samples here is not sufficient to establish beyond doubt whether this latter finding possesses real validity.

In no case did prothrombin titers vary beyond the normal range. However, a detectable decrease was seen in all groups, the greatest change occurring in individuals who received trypsin following surgery. In 9 patients out of 20 receiving trypsin there was a detectable decrease in prothrombin 24 hours after institution of trypsin treatment. During the remainder of the trypsin treatment prothrombin titer was variable. In 4 patients, prothrombin titers rose to or above pretreatment levels over the three day period following the cessation of trypsin treatment.

Prokinase titers were not significantly altered in the presence of trypsin plus surgery, or surgery or trypsin separately. Occasional decreases in antifibrinolytic activity were observed in patients receiving trypsin with and without surgery. These were by no means consistent though in some cases they were pronounced.

Latent coagulable protein followed a pattern similar to that observed for fibrinogen titers. There was a significant rise in patients receiving trypsin following surgery. Neither surgery nor trypsin alone produced significant changes in this entity.

Neither intramuscular trypsin plus surgery nor trypsin nor

surgery individually produced a significant alteration in the spontaneous fibrinolytic activity of the euglobulin fraction. There were isolated instances where precipitous drops in clottable protein of this fraction were seen, indicating that at least in some patients frank fibrinolytic activity was present.

Quantitative determinations of complement titers were made on 16 patients who received trypsin. Of these, six showed a decrease at some point following the institution of trypsin treatment. However, all patients receiving trypsin eventually showed an increase in complement titers to values above control levels. In contrast, only one of 5 patients who did not receive trypsin attained a complement titer above pre-surgical levels.

C reactive protein was elevated in all patients tested. The greatest increases were seen in patients undergoing surgery alone.

Discussion

The fact that intravenous trypsin in relatively high doses renders the blood proteolytic to a degree directly related to the amount of trypsin administered is well known (Innerfield, *et al.*, 1952). Evidence is available that at least under some circumstances trypsin brings about the conversion of profibrinolysin to fibrinolysin (Lewis and Ferguson, 1952; Kocholaty, *et al.*, 1952; Jacobsson, 1953). Findings in this laboratory indicate that trypsin can bring about the conversion of canine profibrinolysin to fibrinolysin both *in vivo* when administered intravenously or intra-arterially but not intramuscularly (Guest and Celander, 1957) and *in vitro* when trypsin is reacted with whole blood (Andrews, 1957). When the relatively small effective doses of intramuscular trypsin acting in most cases at sites distal to the point of injection are considered along with the rate of inactivation of trypsin by serum, it does not appear probable that intramuscular trypsin exerts a proteolytic effect either directly or indirectly in the blood or at the site of inflammation.

Trypsin, as other foreign proteins, may be considered a general stressor. Whether the "antiphlogistic" response is mediated through the adrenals is not clear. Adamkiewicz, *et al.* (1955) reported that trypsin acted to reduce edema in both normal and adrenalectomized rats. However, Hardy *et al.* (1955) observed

that trypsin failed to reduce inflammation in adrenalectomized preparations in which cortisone injections were effective.

The clinical responses to trypsin of the patients studied here have been reported elsewhere (Jackson, *et al.*, 1957). In general, these responses were favorable and corresponded to those obtained by others (Moser, 1957). In view of the clinical findings, it is interesting to note that only minimal changes were seen in most of the blood components studied. Changes in prothrombin, plasma prokinase, antifibrinolysin, and euglobulin lytic activity were such as to indicate that only subtle, if any, changes occurred in the clotting and lysing mechanisms. Elevations were seen in fibrinogen, latent coagulable protein and C reactive protein titers and in erythrocyte sedimentation rate (ESR) and appeared to be in harmony with the concept of a general stress type reaction. Increases in these factors were seen in surgery alone and trypsin alone, as well as in trypsin plus surgery. While both trypsin and surgery individually produced elevations in these factors, surgery plus trypsin did not show as great an augmentation as would have been achieved by simple summation. In general, surgical patients receiving trypsin showed smaller elevations in fibrinogen, ESR, latent coagulable protein and C reactive protein than those who did not receive trypsin.

The increased fluid exchange reported by Innerfield (1956) in areas deprived of normal blood supply by vascular occlusion as well as the carefully controlled permeability studies involving egg white edema reported by Martin, *et al.* (1954), afford evidence that tissue permeability is increased in the presence of even small parenteral doses of trypsin. The increases in circulating fibrinogen as well as in latent coagulable protein observed here may be harmonized with the suggestion by Martin (1957) that trypsin alters the delicate balance between the amount of fibrinogen and related proteins existing in the gel phase of the gel-sol system which he considers to have a great deal to do with permeability in general. If the gel phase were decreased in the presence of trypsin, one might expect a rise in the factors which would normally contribute to it, two of which could reasonably be expected to be fibrinogen and latent coagulable protein. The latter of these may be of more significance than the former since the latent coagulable protein test is a measure not only of fibrinogen but of materials which co-precipitate with fibrinogen under

conditions of physiological pH and ionic strength over a period of 24 hours. An increase in inflammatory reaction as would be seen in surgery could be accompanied by a rise in latent coagulable protein due to its enhanced formation or release from storage depots. Trypsin may also act to depress such enhanced formation or release when given in the presence of surgery or other inflammation-producing stimuli.

While the number of patients studied here is not sufficient to warrant absolute conclusions regarding subtle change or lack of change in many of the factors studied, it is sufficient to permit the conclusion that trypsin in the dosage employed alters neither coagulatory nor fibrinolytic activity, measurable by the tests available.

Summary

1. Studies were made of changes in peripheral blood components and activities in 20 patients receiving trypsin, 15 of whom also underwent surgery, and in 5 surgical patients who did not receive trypsin.

2. Changes in prothrombin, plasma prokinase, antifibrinolysin, and euglobulin lytic activity were such as to indicate that only subtle, if any, changes occurred in the clotting and lysing mechanisms. Elevations in fibrinogen, latent coagulable protein and C reactive protein titers and in erythrocyte sedimentation rate were seen in all groups and appeared to provide support more for the concept of a general stress type reaction to the trypsin than for a direct or indirect proteolytic effect of trypsin.

3. Two new tests are described, one for determination of latent coagulable protein and the other for spontaneous lytic activity of the euglobulin fraction.

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CARBOHYDRATE AND PENTOSE DETERMINATIONS OF SALIVARY GLANDS OF CERTAIN DIPTERA

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In the present study Colorimetric tests were employed to determine the carbohydrate and pentose content of the giant salivary glands of four species of Drosophila and one member of the Phoridae.

Materials and Methods

For analysis salivary glands were isolated from larvae of *Drosophila virilis*, *Drosophila texana*, *Drosophila repleta*, *Drosophila melanogaster*, and *Phoridae*. All of the glands were isolated from healthy, well-fed, mature larvae which had been raised in half-pint milk bottles at a constant temperature of 22–24° C. The standard *Drosophila* culture medium of banana-malt-extract-yeast agar was employed. To insure an adequate food supply, avoid overcrowding, and produce maximum growth the developing larvae were spread to fresh food bottles two or three times during their larval stage. This procedure also gave uniform material for dissection.

The carbohydrates present in the giant salivary glands were determined with Dreywood's (2) anthrone reagent. This is a simple test and very specific for carbohydrates. Morris (4) tested the reagent extensively on a qualitative as well as quantitative basis and confirmed the specificity that Dreywood claimed for it. Test solutions consisting of four ml. were used with eight ml. of the reagent. The salivary glands were completely destroyed by the reagent.

The color developed is measured in a colorimeter against a glucose standard. The range is from 20 to 500 micrograms depending on the filter used. According to Beer's law, the color varies with the amount of carbohydrates present.

Mejbaum (3) reported a colorimetric test for the determination of small amounts of pentose. This test was utilized to establish the amount of pentose present in salivary glands of these Diptera. Two ml. of test solution plus two ml. of reagent are used, and the color produced is measured against an arabinose standard. The range for the pentose is from 1 to 20 micrograms. The proportionality of color obtained in this method is in excellent agreement with Beer's law. Both the carbohydrate and pentose color-measurements were determined in a coleman spectrophotometer using a 630 μ filter.

Preliminary tests, using from five to one hundred pairs of salivary glands per test, indicated that twenty-five pairs of glands per test gave uniform results. Four sets of glands of each species were prepared at the same time to give uniform material for duplicate carbohydrate and pentose determinations. The amounts of carbohydrate and pentose found in these salivary glands and

the per cent of the total carbohydrate which is present as pentose is summarized in Table 1.

TABLE 1

Carbohydrate and Pentose Data

Source of Material	Total Number of Glands Tested	Micrograms Carbohydrate Per 100 Glands	Micrograms Pentose Per 100 Glands	Per Cent of Carbohydrate Present as Pentose
virilis	2000	454	44	10
texana	2000	357	42	12
repleta	2000	241	70	29
melanogaster	2000	163	36	22
Phoridae	2000	308	69	22

Discussion

The results given in table 1 indicate interesting differences in the salvary gland analysis. As would be expected, the overall results tend to indicate species differences. Not only is there a difference in the total carbohydrate content but also in the pentose content. As far as the *virilis* and *texana* analyses are concerned this difference is not very great. Although the total amount of carbohydrate present is somewhat lower in *texana* than in *virilis*, the per cent of carbohydrate as pentose is almost the same. If all of the pentose is present as a constituent of nucleic acid, then this would seem quite constant for these two closely related species. The amount of total carbohydrate is much less in *repleta*, but the per cent of pentose is significantly higher in this species than in *virilis* and *texana*. The per cent of pentose in melanogaster is approximately the same as that of *Phoridae*, but, as the glands of the latter are much larger, the total carbohydrate content is also larger.

Although members of the virilis group, morphologically *virilis* and *texana* are quite different, and according to Blumel (1) it is possible this morphological difference resides in the amino acid components of the genes of these two species. Certainly, the results of the sugar analyses seem to be much more constant for these two species. As pointed out above, the difference in the per cent of pentose in *virilis* and *texana* is almost negligible. The higher per cent of pentose in *melanogaster*, *repleta* and *Phoridae* is not surprising, as the contents of the salivary glands of these

three Diptera are much more viscous, and viscosity is often associated with the amount of nucleic acid present. In *virilis* salivary glands pentose amounts to 1.9 per cent of the total weight of these glands. It is not inconceivable that all of this is present in the form of nucleic acid. If this is true, then the closer species relationship of *virilis* and *texana* would reside in the nucleic acid moiety rather than in the overall amino acid content.

Summary

In the experiments described, salivary glands of four species of Drosophila and one member of the Phoridae were analysed for their carbohydrate and pentose content. In 100 pairs of salivary glands of *virilis* analysed, 10 per cent of the total carbohydrate was in the form of pentose. In *texana* 12 per cent of the total carbohydrate constituted the pentose fraction. The other two species of Drosophila analysed had a much higher percentage of pentose. *D. melanogaster* showed that 22 per cent of the total carbohydrate was in the pentose form. *D. repleta* had 29 per cent of its total carbohydrate in the form of pentose. Phoridae, a non Drosophila had 22 per cent of its total carbohydrate in the form of pentose.

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PURIFICATION AND CHARACTERISTICS OF A VIRUS ISOLATED FROM HUMAN LYMPH NODE INOCULATED MICE[a]

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Over a period of years Hodgkin's disease has repeatedly been looked upon as possessing certain clinical and pathological characteristics that suggest that it would be one of the more promising human malignancies to study in search of possible microbial etiology. At first, the main efforts were directed toward demonstrating a causative bacterial agent. Although several types of bacteria were isolated from ground Hodgkin's disease lymph nodes, they were not considered to be specific since similar bacteria have been found in normal lymph nodes (6, 7). Gordon (6) after five years of research on Hodgkin's disease, suggested that a virus agent should be considered as a possible cause of this disease. Since then several reports on Hodgkin's disease studies, from a virus point of view, have appeared. They have been reviewed critically by Bostick (4).

In 1948 Bostick (3) reported that the serial passage of filtrates of involved tissues in Hodgkin's disease in embryonated chicken eggs resulted in a slightly increased egg mortality over the egg series inoculated with similarly treated non-Hodgkin's disease lymph node material. In later studies this chicken egg material, as well as original Hodgkin's disease lymph node homogenates were passed blindly in newborn mice in an effort to detect any responses that would suggest the presence of a virus in the Hodgkin's disease material.

After from six to nine blind intracerebral passes, the mice from three of the series inoculated with Hodgkin's disease material developed a fatal encephalitis. This disease was transmissible, was lethal, and the brain material possessed all the biological characteristics of a virus (5). The purpose of this report is to present certain of the data showing the method for the physical purification of this virus, its electron microscopic appearance and some of its reactions to pH and temperature conditions.

Experimental

Material: The virus used in these studies was virus "P" that was isolated following serial intracerebral passage in newborn Princeton strain albino mice of fresh, ground material of a histologically classical Hodgkin's disease granuloma-filled lymph node. After six blind passes of harvested mouse brain material, the baby mice showed some paralysis and mortality, and by the eighth passage a well-established fatal disease developed which has been passed in these laboratories for 2 years. The virus has been adapted after repeated trials to weanling mice. The material used for this purification was the infected brain harvested for the 18th serial intracerebral passage. At that time the L.D. 50 of the virus was 10–5.2.

Purification Method

The method adopted for purification in this report is a modification of a butanol extraction procedure developed by Bachrach & Schwerdt (1). Because of the great amount of lipid in brain tissue, it is quite difficult to separate virus from it by differential centrifugation. Some of these lipids can be successfully removed by ether, but considerable virus may be lost in the gel which develops at the interface of the aqueous and ether layers (1).

Table 1 is a flow diagram of the different steps in the purification procedure. All steps are carried out at 4° C. Essentially, the brain tissue is homogenized and a 10 per cent suspension in 0.1 M. NaCl is adjusted to pH 4.5, with 2 M. Acetic Acid. The precipitate is resuspended in veronal acetate buffer at pH 9.0, 1 volume of cold N-butanol added and then emulsified for 0.5 hours in a Waring blender. The resultant emulsion is centrifuged at 4000 RPM for a half hour, and of the four distinct phases formed the aqueous is saved, and the gel, butanol and pellet discarded. The aqueous phase is again emulsified for 0.5 hours with 1 volume of N–butanol as described above. From that procedure the aqueous phase is saved, centrifuged for 2 hours at 78,000 g and the resultant pellet resuspended in 0.1 M. NaCl. The suspension is cleared by a final centrifugation at 9000 g for 10 minutes and the supernatant retained for purification evaluation.

Biological Evaluation of Purification

The supernatant of the final preparation was tested for its 50 per cent lethal dose (L.D. 50) after 21 days in weanling mice, and this was compared with the L.D. 50 of the original tissue homogenate which served as the source of the purified material. In order to evaluate further the degree of purification, the "specific" L.D. 50 was determined, *i.e.* the negative logarithm of the concentration of protein in grams per ml. which is lethal within 21 days for 50 per cent of the inoculated mice. Nitrogen analysis was carried out by sealed tube digestion with sulfuric acid, mixing the digest with Nessler's reagent with the aid of a magnetic stirrer, and reading the resulting color complex photometrically at 480 mu. (8). Samples were run in duplicate with appropriate blanks included in each set of analyses.

Comparison of the "specific" L.D. 50 of the original homogenate with that of the purified material affords an indication of the relative concentration of virus protein as compared to other types of proteins present in the homogenate.

TABLE 1

Flow Diagram of Purification Procedure

Homogenate*

↓ Adjusted to pH 4.5 with 2M acetic acid

Precipitate (Protein and Virus)

↓ Resuspended in veronal acetate buffer pH 9.0, stirred 0.5 hrs. with 1 volume n-Butanol, and centrifuged.

Butanol Phase	Gel	Aqueous phase
↓	↓	Stirred 0.5 hrs. with 1 vol. butanol and centrifuged; discard butanol phase and gel.
Discard	Discard	↓
		Aqueous phase
		78,000 g for 2 hrs., 4° C.
		↓
		Pellet
		Resuspend in 0.1 M NaCl; centrifuged 9,000 g for 10 min.
		↓
		Supernate

* 10 per cent suspension of mouse brain tissue in 0.1 M NaCl; prepared in a Waring blender homogenizer.

The precipitation procedure was carried out on 3 separate occasions with resultant highly comparable results. In table 2 the detailed results of 2 of these experiments are presented. These show that with the purification procedure employed, only 6 per cent of the infectivity observed in the original homogenate was recovered. However, comparison of the nitrogen content of the initial and final preparations showed that from 95–97 per cent of the nitrogen (going from 2500 ug. N/ml. to 100 ug. N/ml.) was removed by the purification, which accounts for the increased specific infectivity of the purified material by 250 and 315 times respectively over that of the original material.

Electron Microscopy

For these studies aliquots of the final supernatant were dialyzed in a collodion membrane against 0.1 M ammonium aletate for 24 hours at 4° C.

TABLE 2

Yields and Specific Infectivities of Virus Following Butanol Extraction and One Cycle of Differential Centrifugation

Experiment No.	LD_{50}*		Recovery of Infectivity Per Cent	Specific LD_{50}†		Increase in Specific LD_{50}
	Tissue Homogenate	Final Preparation		Tissue Homogenate	Final Preparation	
I	5.6	4.4	6	7.3	9.7	250 x
II	5.9	5.0	13	7.7	10.2	315 x

* Negative log of the dilution lethal for half the inoculated mice within twenty-one days.
† Negative log of the concentration of protein in gm./ml. lethal for half the inoculated mice within twenty-one days.

Polystyrine latex particles (260 mu. diam.) were added, the resultant mixture sprayed onto collodion-coated grids (2) and shadowed with uranium at an angle of approximately 1:5 prior to examination.

Electron micrography (Figs. I and II) illustrates the typical appearance of the experimental and control material respectively. In figure I, two different-sized, approximately spherical-shaped particles are seen to predominate, one about 31 mu. in diameter and the other about 17 mu. In the electron micrograph of a preparation from normal mouse brain (Fig. II) the larger particles observed with infected material is no longer seen, the predominant particle now being one which measures about 17 mu. Since these smaller particles occurred in both normal and infected materials they have been regarded as normal components of mouse brain; especially since no infectivity was ever noted with normal preparations, whereas the infected concentrates produced paralysis in mice at dilutions of 1:10 (5) to 1:10 (6).

Effects of Temperature and Hydrogen Ion Concentration on the Purified Virus

The purified concentrated virus was suspended in veronal acetate buffers of various pH ranges in order to study the stability of the virus at various hydrogen ion concentrations. All effects were observed at only one temperature, namely 4° C.

As will be seen from table 3, the pH's checked were 1.9, 4.6, 7.3, 8.1 and 9.3. The time that they were exposed to these was also varied, namely 1 hour and 1, 3 and 7 days. After being exposed at 4° C. for the time and pH indicated, samples of each virus suspension were removed for bio-assay.

In the range of pH 4.6 to 9.3 the virus was quite stable. At pH 1.9 the infectivity was seriously reduced, even though in this experiment a complete endpoint was not reached since the lowest dilution tested was 1:1000. The marked drop in infectivity at the end of 1 hour at pH 7.3 was surprising and is unaccounted for, although it has been observed in other pH stability studies with crude poliomyelitis virus suspensions (1).

Temperature inactivation studies were done at pH 7.4. The virus was stable at 37° C. for 4 hours (the longest period tested). However, at 56° C inactivation was quite striking even within 15 minutes, the infectivity being reduced by more than a thousandfold in that time.

Discussion

More detailed studies of the previously reported virus (5) isolated from mice inoculated intracerebrally with Hodgkin's disease lymph node material have been performed. In preparation for broad serologic tests it became desirable to develop methods for purifying the virus to the highest degree. The method developed by Bachrach and Schwerdt (1) was modified to fit our special needs. It is based on repeated cold N-butanol extraction followed by final centrifugation at 78,000 g for 2 hours. L.D. 50 and "specific" L.D. 50 studies demonstrate that the procedure is very effective, for it is capable of removing 97 per cent of the extraneous protein (nitrogen) while increasing the specific L.D. 50 of the purified material up to 315 times that of the crude homogenate.

Electron micrographs reveal a spherical 31 mu. diameter particle that is found only in the infected mouse brain material. A 17 mu. diameter particle encountered in both infected and non-

FIG. 1. Purified virus preparation from Hodgkin's inoculated mouse brain. Scattered larger particles 31 mu. diameter were present only in the experimental material. Magnification X 60,300.

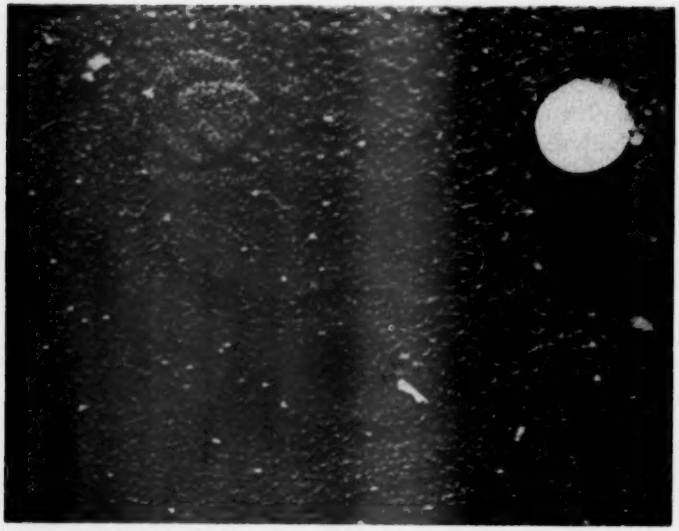

FIG. 2. Purified preparation from uninoculated normal mouse brain. Note the absence of the 31 mu. particles and the presence of fine 17 mu. particles in the background which are present in both the control and experimental tissues. Magnification X 60,300.

TABLE 3

pH—Stability of Virus Infectivity After Exposure to Various Hydrogen Ion Concentrations

	LD_{50}*				
Time/pH	1.9	4.6	7.3	8.1	9.3
1 hr.	< 3.0	4.7	3.4	4.6	4.7
1 day	< 3.0	4.1	5.0	5.0	5.0
3 days	< 3.0	3.5	4.6	4.6	4.5
7 days	< 3.0	< 3.0	4.6	4.4	4.4

* Negative log of the dilution lethal for half the inoculated mice within 21 days.

infected animals does not appear to be related to the disease produced in the mice by the serially passed Hodgkin's disease material.

These experiments were designed in the main to purify the virus and to study a number of its physical properties. The size and shape of the purified agent were studied by means of the electron microscope. Additionally, temperature and pH stability

studies of the purified agent, subject to fewer variables than similar tests with crude material, could be carried out in a more reproducible fashion and with results more significant with respect to the nature of the virus itself. In this report no data is being presented to relate the virus isolated and observed to the Hodgkin's disease lymph node material which was originally inoculated into these experimental mice (see Bostick and Hanna (5)). With the use of the purified virus, improved studies of antigen and antibody reactions can be anticipated and are being performed.

Summary

A purification procedure is described which has been successfully used to isolate the virus present in brains of mice originally inoculated with Hodgkin's disease lymph node material. It is based on repeated N-butanol extractions at controlled pH and temperature with a final differential centrifugation cycle. The procedure removes about 97 per cent of the original protein and also some of the virus. However, it removes relatively much more non-virus protein than virus protein as demonstrated by the fact that the purified material has a specific L.D. 50 titer approximately 300 times that of the crude material.

Electron micrographs of infected mouse brain material revealed a very clean preparation that contained spherical particles 31 mu. in diameter, which were not present in similarly prepared material from control animals.

The infectivity of the virus concentrate was quite stable in the pH range 4.6 to 9.3, while at pH 1.9 it was seriously reduced. The virus was highly stable at 37°C. for 4 hours but at 56°C. its infectivity was reduced a thousandfold within 15 minutes.

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THE EFFECTS OF A THORAZINE-CENTERED TREATMENT PROGRAM WITH PSYCHOLOGICAL EVALUATIONS[a]

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During the past three years Thorazine, or chlorpromazine, has become one of the most highly valued drugs in use for the treatment of mental illness. This study was made in an attempt to identify psychological variables which may be subject to alteration by the action of Thorazine. It is also hoped that the paper will help other research workers in developing hypotheses which may be tested by empirical methods. Because of a limited supply of Thorazine, difficulty in obtaining placebos, and limited professional help, it became necessary to limit the research to the study of a single group. In an attempt to effect sufficient change for measurement the authors became interested in studying the effects of Thorazine treatments under circumstances which should offer maximum effectiveness. A total-push program was selected. The assumption accepted for this project was that Thorazine can lower the reinforcement properties of emotional disturbances and disequilibruim, thus presenting an opportunity for the patients to learn and reinforce more beneficial adjustment technics. Also, by reducing the emotional element, the value of the intellect as a reinforcement agent can be increased. The assumption recognized that the surrounding conditions must be conducive to learning and exercising better adjustment reaction patterns.

These assumptions were suggested from a review of several reprints furnished by the Smith, Kline and French Laboratories. Delay and co-workers (1952) found the drug useful with states of excitement, depression, anxiety, etc., but their stressing of "behavioral and mood disorders" suggests that their criteria were in terms of observable changes in symptomatology. They also observed that external excitations seemed to reach the patient in subdued form and that indifference was paramount, that the patients seemed to be divorced from their environment. Staehelin

and Kielholz (1953) suggest that the drug may allow a breakthrough of the vicious circle cause by psychic tension, sympathetic dystonia, etc. Lehmann and Hanrahan (1954) in a very small scale investigation, found that only tapping speed seemed to undergo impairment from Thorazine. They assume that tapping speed is dependent upon inner motivation without external stimulation. The considerations suggest the need for an increase of constructive external stimulation in an attempt to counteract the possibility that the patient may be come satisfied with alleviation of personal discomfort and indifferent to try out new adjustment responses in reality. Further, these articles suggest that Thorazine has a depressing effect upon neural functions, especially the emotions. If Thorazine reduces the value of the emotions as a reinforcing agent to pathologic functions, it might follow that the intellect increases in value as a reinforcing agent. Thus, should Thorazine allow one to give up pathological processes, learning theory dictates that new technics must be made available from the environment and that the environment allows the person to practice and reinforce the new learning.

Procedures

Fifteen female patients participated in a special project which represented an attempt to provide a therapeutic community in which conditions were made as favorable for improvement as was practical. Eighty per cent of these patients were chronic schizophrenics. There was one manic and two acute schizophrenics.

In order to meet personal needs of the patients, three didactically oriented group psychotherapy and two special recreation sessions were held each week. In addition, the patients were allowed to attend regular hospital-wide recreation (usually consisting of one dance and one movie each week with occasional trips to the little store and other smaller activities), were encouraged to have sessions of their own before retiring to bed, and were occasionally given individual counseling by the ward physician or a psychologist. Six patients were allowed to work at jobs of their own choice about the hospital, after several weeks of active treatment. After the first week the women were quartered on the best hospital ward available to white female patients. Ward visits were made occasionally by a psychologist, the hospital chaplain, and a social worker, during which times the patients were encouraged to discuss personal problems. Ward personnel were oriented as to the nature of the project and special roles that they might play. Before any patient was released from the hospital, a counseling session was held by the ward physician or a psychologist with the patient's relatives.

The program was inadvertently divided into two phases of treatment. The

first phase included three and one-half weeks of Thorazine treatments[d], after which the hospital's drug supply was exhausted, plus four and one-half weeks of psychotherapy. Thorazine was administered by intramuscular injection for the first ten days, starting with 50 mg. the first day and increasing the dosage by 50 mg. each succeeding day until the best maintenance dosage was discovered for the individual patient. Patients were held on this dosage, which did not exceed 300 mg. per day for any one patient, until they were changed to equal oral dosages at the end of the first ten days. Having had one orientation session before the drug was first administered, the patients began their participation in group psychotherapy and group recreation at this time. Group psychotherapy was continued for four and one-half weeks, the termination of which marks the end of the first phase of treatment. Recreation sessions represented the only organized activities during the next two weeks. At the end of this two weeks period seven of the nine patients remaining in the hospital began the second phase of Thorazine treatment. This phase, which lasted for five weeks, included no psychotherapy, but all privileges were continued and patients attended small group recreation activities. Psychological evaluations were made on ten patients participating in the special project. Tests were administered one to two weeks before and four days to two weeks after treatment.

I. General Subjective Results

At the end of treatment nine of the fifteen patients were allowed to leave the hospital. Seven of these were still at home at the end of an eighteen month follow-up period. Two patients returned after thirteen or fourteen months. Follow-up psychological testing suggested that one was in better mental condition than when she left. The other patient was in approximately the same condition, except that her behavioral controls were somewhat more relaxed. Three other patients went home at a later date, after continued Thorazine treatment. These patients were still home at the end of a one-year follow-up period.

For the first phase of treatment, a rating scale, consisting of twelve items, was filled out once each week by ward personnel (nurse technicians). Item twelve, "mood," revealed a mean increase of 1.1 points, significant to the 1.0 per cent level of confidence. Only two of the fifteen patients lost ground, whereas three of the four patients who fialed to improve had maximum ratings initially. Significant change in one of twelve items is expected on the base of chance alone, but improvement of mood corresponds with behavioral observations. The group as a whole exhibited greater social interaction, management problems almost

disappeared and the ward reflected a more pleasant and cooperative atmosphere.

II. Psychological Evaluations

The research was designed initially to include full battery testing on twelve subjects, but two of these subjects were removed from the program before post-testing was done. Due to an error in communication they were mistaken for the two subjects who were untestable initially.

Of the ten subjects receiving psychological evaluations, eight were discharged immediately after post-testing and the remaining two were judged to show marked improvement. One was discharged less than two months later. Eight of the nine patients discharged remained home for approximately one year, six for eighteen months and four had not returned thirty-four months later. These results suggest that psychological testing should be able to sample change occurring in the subjects during treatment.

a. Psychometric Evaluations

Nine Wechsler-Bellevue sub-tests and the Graham-Kendall Memory-For Designs were administered before and after treatment. Five patients received post-testing and were discharged after the first phase of treatment. The remaining five subjects were re-tested after the full treatment program. The test re-test method was used. Wechsler's Form I and II were each administered to five subjects, but no apparent differential effects were reflected in the results.

Results are presented in table 1. There is some question about meeting the assumptions for using small sample t-ratios for evaluating changes, but it is believed that the assumptions can be defended well enough to use the results as the basis for formulating tentative hypotheses.

Table 1 shows that the group as a whole increased their mean Full Scale I.Q. by a statistically significant 10.5 points which may be largely due to the more significant mean increase of 16.0 Performance I.Q. points. At best, the mean increase of 4.3 Verbal I.Q. points is "provisionally significant" statistically. The Graham-Kendall test[e] reveals a mean improvement of –3.0 points, which is also "provisionally significant." Specific Wechsler-Belle-

TABLE 1

Results of Psychometric Evaluations of Ten Patients, Before and After Thorazine-Centered Treatments

Test	Mean Gain	Small Sample t	Probability
Information	1.0	2.2	.05
Comprehension	0.4	0.4	.70
Digit Span	0.7	1.1	.30
Arithmetic	1.1	1.6	.15
Similarities	0.6	0.9	.39
Picture Arrangement	3.4	4.8	.01
Picture Completion	2.4	3.0	.02
Block Design	1.6	3.5	.01
Digit Symbol	3.5	4.0	.01
Verbal I.Q.	4.3	2.0	.08
Prorated Performance I.Q.	16.0	8.4	.01
Prorated Full Scale I.Q.	10.5	5.8	.01
Graham-Kendall	−3.0	2.0	.08

vue sub-test results are presented in table 1 for the reader who may wish to formulate tentative hypotheses for future research.

These results reflect significant improvement in the ability to apply one's mental resources to the task of coping with the immediate environment and in the ability to overcome interference from mental and/or emotional disturbances. The fact that improvement is shown in all test functions studied suggests that the subjects in this study generally experienced an increase of mental efficiency during their participation in the treatment program. Several more or less basic functions seem to be involved but the limitations inherent in the present design do not seem to justify the use of additional space for detailed analysis of table 1.

b. Qualitative Evaluations

The Draw-A-Person, Facial Expression Pictures (a projective test devised by J. E. Nickols, Jr..) and Rorschach offer qualitative evaluations. Because the results of these tests do not appear contradictory and the Draw-A-Person and F.E.P. were not administered after the second phase of treatment, only analyses of Rorschach results are reported in this paper. Rorschach results were omitted for one subject because of questionable validity due to the use of a substitute examiner for post-testing.

Nine Rorschach evaluations were made after treatment, four after the first phase and five after the second phase. Chi square

evaluations were made on scoring categories of location, determinants, and reaction times, plus A and H per cents. The frequency of significant changes, of the total number of comparisons made, may be expected on the basis of chance alone. However, nine subjects greatly limit the sensitivity of chi square and it may be realistic to use probabilities that better the eight per cent level of confidence for formulating tentative hypotheses for future research. Of the main determinants, only Form responses decreased significantly. Acromatic color responses were the only additional determinants to decrease significantly. Animal responses increased to the eight per cent level of confidence, whereas human responses did not change. Average reaction time to color, acromatic and all cards each decreased to the eight per cent level of confidence. The sum of main determinants did not change, whereas the sum of additional determinants decreased significantly. Also, main determinants were each observed for the strength and direction of change only for subjects who did not show the same post-testing scores as pre-testing scores. F and C′ show decreasing trends, whereas FM tends to increase.

Rorschach analyses most noticeably suggest that the trends of change occurring during the treatment were highly individual, depending upon the subjects' basic personality structures, need patterns and attitudes. This hypothesis is strongly supported by the fact that only two patients failed to show marked variation between pre- and post-Rorschach scores, even though there is a general lack of consistency in direction or magnitude of change among the scoring categories. The hypothesis also seems to gain some support from the observation that, while F scores decreased, no other types of main responses changed significantly.

These results also suggest that, for the most part, the patients experienced no significant loss of mental drive, although at the end of treatment it may have become redirected, or at least released by somewhat different means or different proportions of the same means. Except for a possible trend toward becoming more aware of impulses toward immediate gratification, there is no evidence that mental drive had become redirected into self-understanding.

Certain trends are suggested from a more strictly clinical analysis of Rorschach records as a whole. Where possible, clinical interpretations were checked for consistency against a compila-

tion of various scoring categories. These results most prominently suggest that acute or exaggerated disturbances had been alleviated to some extent by the end of treatment; but at the same time, there was a decisive lack of improvement in the basic illness and a noticeable trend toward the patient's chonicity becoming more apparent.

This conclusion is supported by several trends. Verbalizations show less doubt, perplexity, confusion and/or irrelevant discussions of percepts, but more specific, careless and/or indifferent reactions to their perceptions, even where the content is obviously characteristic of the sick mind. There is a general trend toward a lessening of emotional disturbance, particularly in relation to environmental stimulation. This does not seem to be accompanied by trends towards increases in self-understanding, significant or meaningful relationships with others, subjective adjustments to anxiety or adequacy of conscious controls. There seems to be some trend toward increased withdrawal and less concern over the self or environment. The treatments seemed to have had some effect upon the patient's reactions and behavior but little effect upon the basic illnesses or contents of perceptions.

Klopfer,[f] after a preliminary survey of the data, concluded that there seems to be an increase of "spontaneity" in the reactions of the patients but also "in their carelessness in the use of the stimulus material." It seems as though the patients became more inclined to play around with and/or make feeble trial and error attempts at testing reality. This lessened need to avoid reality testing may be associated with the noticeable trend toward improved accuracy of impersonal perception, but there also seems be a trend away from obvious or conventional responses and no apparent change in the accuracy of general perception.

Prognostic ratings failed to reveal statistically significant change. A slight trend is noticeable toward improvement of prognosis, but negative trends were almost as apparent.

Summary and Synthesis

The study presented in this paper represents an attempt to identify psychological variables which may be subject to modification through the use of Thorazine-centered treatments. The authors selected the use of a "therapeutic community" in an at-

tempt to allow optimal external stimulation and adequate opportunity for Thorazine to effect change. The project had much to be desired, the most outstanding weaknesses being the small number of patients studied, lack of adequate controls and lack of a control group, but it is hoped that the paper contributes tentative hypotheses which may be followed up in future research.

The Thorazine-centered total-push program helped nine of the fifteen patients to recover sufficiently to be allowed to leave the hospital shortly after the project ended. Only two had returned at the end of an eighteen month follow-up period; neither patient appeared to have lost ground psychiatrically. The group as a whole reflected improvement in terms of mood, social interaction, ward atmosphere and management problems.

The results of psychological testing suggest definite improvement in mental efficiency. The patients are better able to set their mental resources and intellectual achievements to the task of coping with the immediate environment. The tendency for only performance tests to reflect significant improvement, along with Rorschach indications that mental drive tends to remain constant but may experience redirection, suggests that greater efficiency is obtained through an increase in ability to overcome interference from mental and/or emotional disturbances and in psychological freedom to interact with the environment.

The fact that verbal tests and Rorschach content seem hardly affected suggests that there is no change in basic content of the illness. The patients apparently experienced an alleviation of their overactive and/or acute disturbances but their basic illnesses and/or chronicity may have become more readily revealed.

The increase in efficiency seems to be manifested by increased ability to cope with the environment, increased spontaneity and interaction, but does not tend to be accompanied by better emotional or more meaningful relationships with the environment. Even though there seems to be a decreased awareness of pressure or threat from the environment, there also seems to be an increase of indifference or carelessness in dealing with the environment, and less concern with one's personal illness.

All tests reveal highly individualized changes among the subjects, suggesting that the effects of Thorazine are largely dependent upon the more basic personality structure and ego values. There are suggestions of decreased ego defensiveness, but there

seems to be no apparent change in the weakness or immaturity of the ego structure. The mental or intellectual functions which are subject to change also show individualistic trends.

From this study, it is impossible to identify differential effects of various activities and treatments of the program. This must be left to future research. The results suggest that research should study methods of using Thorazine treatment, as well as what effects may be characteristic of the drug. It is very difficult to single out specific psychological functions which may be altered by appropriate uses of Thorazine treatment, because of the danger of interpreting the results out of context. It is suggested that the reader extract his own specific hypotheses from the considerations given in the text of the paper.

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[a] A preliminary report on this work has been given by Covington and Nichols (1954). The authors are deeply indebted to Dr. Bruno Klopfer, University of California, and to the students of an advanced seminar under his direction, for scoring and interpretations of pre- and post-records of five subjects. The reports and Dr. Klopfer's communications offered valuable guides for many interpretations of Rorschach results. The authors are also indebted to Leigh Peck, Ph.D., Associate Professor, The University of Texas, for her sponsorship of this publication and her interest in reviewing the Rorschach and other analyses. In addition they also wish to thank all hospital personnel who assisted in this project, particularly G. J. Browning and James McElroy, as well as Ruth Rose and the secretarial staff at the Hertzler Clinic, who helped prepare this manuscript.

[b] Now at the Austin State Hospital, Austin, Texas.

[c] Now at the Hertzler Clinic, Halstead, Kansas.

[d] Drugs for the first phase of treatment were furnished by the Smith, Kline and French Laboratories.

[e] In the scoring of this test, "forgets" were arbitrarily assigned values of 1.0 and "can't do" responses 2.0, unless an observation of partial reproductions suggested trends toward reproductions that if completed would have received other score values.

[f] Personal communication.

Subsequent Study

Nichols, J. E., Jr.: An Exploratory Investigation Into The Effects of Thorazine Upon The Mental Test Scores of Chronic, Hospitalized Schizophrenics. Unpublished Doctoral Dissertation. University of Denver, 1957.

Accepted for publication November 16, 1957.

Postscript: In a highly controlled subsequent study, Nickols (1957) has attempted to test some of the findings of this paper. Generally, Nickols reports that his more chronic schizophrenics failed to show conclusive difference for Thorazine and Placebo treatments, but that certain rather definite trends are suggested in the results. Thorazine seems more effective than Placebo in improving mental efficiency, for the performance part of the Wechsler-Bellevue Scales. Results of Rorschach and Facial Expression Picture Evaluations tend to compare favorably with corresponding results reported in the present study.

EFFECT OF HUMAN BLOOD SERUM ON TISSUE CULTURES I. SOME PROPERTIES AND SPECIFICITY OF TOXIC HUMAN SERUM, AND ITS INTERACTION WITH STRAIN L CELLS

SERGEY FEDOROFF[a]

In an attempt to detect substances specific to schizophrenia, blood serum was examined for its effect on tissue cultures of strain L cells (4). In addition to serums from schizophrenics, serums from non-schizophrenic mental patients, surgical patients and healthy persons were also tested (5). Within each group there were found individuals ranging from those having completely non-toxic serum to those with highly toxic serum. However, the mean degree of blood serum toxicity did differ from one group to another, and was highest in the group of schizophrenic patients. The results implied a relationship between high serum toxicity to strain L cells and schizophrenia. The results also raised the questions: why does the blood serum toxicity vary from person to person, and, is it related to acquired or inherited factors? To investigate these problems, persons with blood serum of the same toxicity could be grouped disregarding clinical diagnosis, and a search could be made for common denominators within the groups. Thus far in this investigation serum toxicity has been measured in terms of cell reaction or cell death, both of which could be caused by many agents. Therefore, before attempting to correlate serum toxicity with specific physiological or pathological states, it seems necessary to establish whether the toxicity of the blood serum from any two individuals is caused by the same substance or substances.

The present investigation deals with some properties of toxic substances in human blood serum, and their interaction with, and effect on, strain L cells.

Experimental Procedures and Results

1. *Method for determination of blood serum toxicity.* The culture method used and the procedure for testing serum has been published elsewhere in detail (4). Briefly, strain L cells are grown directly on glass in Leighton

tubes. When cultures have grown to a suitable density the medium is removed and replaced by the serum to be tested, diluted 1:1 with Hanks' balanced salt solution (8). To protect the cultures from possible bacterial contamination 200 units of sodium penicillin are added. The pH is adjusted to 7.4–7.6 with sodium bicarbonate, and the cultures are incubated at 37° C. for three days. They are examined microscopically each day and the toxicity is graded from zero (0) to 4 plus (++++), on the basis of the degree of cell destruction. The final degree of toxicity is calculated as the mean of the three daily readings. In earlier investigations it was found that a large percentage of schizophrenic patients have toxic blood serum (4,5); therefore the toxic blood serums for the experiments described in this paper were largely obtained from schizophrenics.

2. *Determination of some properties of toxic serum.* To gain insight into the nature of the substances which make some human blood serums toxic to cultures of strain L cells, such serums were subjected to various conditions. It was found that toxic serum could be stored for at least 4 days at 4° C., for 126 days at —20° C., or in the lyophilized state, without significant changes in the degree of toxicity (Table 1).

TABLE 1

Effect of Storage on Serum Toxicity

Storage Temperature	Treatment Before Storage	No. of Samples	Duration of Storage (Days)	Serum Toxicity Before Storage Mean ± S.E.	Serum Toxicity After Storage Mean ± S.E.	P
+ 4° C.	....	19	4	2.9±0.3	2.4±0.3	>0.05
—20° C.	....	32	8–126	3.8±0.1	3.5±0.2	>0.05
+ 4° C.	lyophilization	5	2	2.0±0.9	2.0±0.6	>0.05

When toxic serum was incubated at 37° C. there was a gradual reduction in toxicity, depending on the duration of incubation (Fig. 1), with complete detoxication requiring approximately 48 hours. At 56° C., however, serum was detoxified in 30 minutes (4). Toxicity could also be reduced or completely eliminated by diluting the serum with balanced salt solution (BSS) (Table 2).

When serums were shaken on a mechanical shaker for 24 hours at +4° C. there was no change in toxicity, but when the shaking was done at room

TABLE 2

Effect of Dilution on Toxicity of Human Blood Serum

No. of Serum Samples	Dilution (Serum : BSS)	Degree of Serum Toxicity Mean ± S.E.
5	1:2	4±0
5	1:4	3.6±0.24
5	1:8	2.8±0.37
5	1:16	1.8±0.37
5	1:32	0.2±0.2

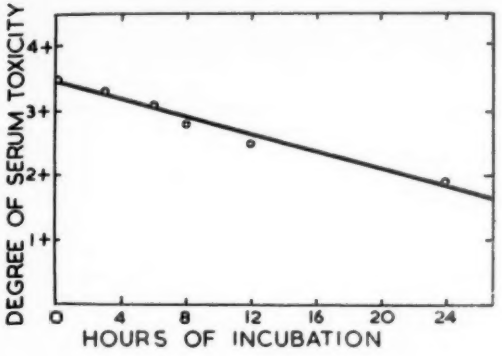

FIG. 1. Effect of duration of incubation (37° C.) on toxicity of human blood serum. Each point on the curve represents the mean toxicity of 10 serums.

temperature a decrease in toxicity was observed. It seems likely that the decrease at room temperature was due to the effect of heat radiated from the shaker.

To determine whether the toxic substances are large or small molecules, serums from 16 schizophrenic patients and from 4 non-schizophrenic mental patients were dialyzed against balanced salt solution. Under aseptic conditions, 4 ml. of each serum were dialyzed for four days at 4° C. and pH 6.8, against 1000 ml. of Hanks' balanced salt solution, changed twice daily. Control samples of the serums were kept at 4° C. for the same length of time. The dialyzed and control serums were then tested for toxicity on cultures of strain L cells. No significant differences in toxicity were detected (Table 3).

3. *Effect of toxic human serum on the morphology of strain L cells.* In an attempt to learn how strain L cells are killed by toxic human serum, the following experiments were made. Cultures to which toxic serum had been added were fixed in 10 per cent neutral formalin solution after 2, 4, 8, 15, 30 and 60 minutes incubation, in order to observe microscopically the various stages leading to cell destruction. In another experiment cells were grown

TABLE 3

Effect of Dialysis on Serum Toxicity

	Degree of Toxicity		
No. of Samples Dialysed	I Serum Stored at −20° C. Mean ± S.E.	II Serum Kept at +4° C. for 4 Days Mean ± S.E.	III Serum Dialysed at +4° C. for 4 Days Mean ± S.E.
20	2.95±0.37	2.50±0.37	2.55±0.25

in perfusion chambers on the warm stage of a microscope. One cell was selected, under oil immersion, and photographs of the cell were taken every two minutes for half an hour. The growth medium was then removed and toxic human serum, diluted 1:1 with Hanks' balanced salt solution, was added. Photographs were again taken every 2 minutes for two hours. Combining the observations made on the material prepared by these methods, the following morphological changes were recorded: A few minutes after the addition of toxic human serum to a culture of strain L cells, the cells rounded up (Figs. IIb and IIIb), cytoplasmic blebs appeared on most cells (Fig. IIb), the cytoplasm became granular (Figs. IIc and IIIc), and the cells disintegrated (Fig. IId). At the same time the nuclei became round (Fig. IIId) and the nuclear membranes thick and dense (Fig. IIId). The nucleoli, barely visible at first figures IIIa and IIIb), became prominent for a time (Figs. IIIc, IIId, and IIIe), and when the nuclei decreased in size (Figs. IIIe and IIIf), the nucleoli disappeared (Fig. IIIf). During this time the cytoplasm became translucent and surrounded by a well-delineated border (Figs. IIIe and IIIf). The granular portion of the cytoplasm was pushed to one side of the cells along with the nuclei (Figs. IIIe and IIIf). At this stage many cells became detached from the glass surface and floated free in the medium. The duration of the process varied with the serum used, but was approximately 30 minutes to 2 hours, at which time many or all cells were dead. According to Lettré and Schleich (15), cytoplasmic bubbling which is caused by the addition of Victoria Blue to cultures, can be suppressed by adenosine triphosphate (ATP). Cells affected by toxic human serum also showed some activity of the cytoplasmic membrane, *i.e.*, the production of bleb-like structures which constantly changed in size and position. Therefore the sodium and potassium salts of ATP, in the concentrations used by Lettré, were added to strain L cells exposed to toxic human serum. In one experiment ATP was added to the cultures together with the toxic serum, and in a second experiment the cells were pre-treated with ATP before the addition of toxic serum and more ATP. No inhibition of the formation of blebs was observed in either experiment. As a matter of fact, cells treated with ATP plus toxic serum seemed to show bleb formation for a longer time than usual.

4. *Effect of toxic serum on epithelium-like and fibroblast-like cells.* The foregoing experiments were performed on strain L cells. These cells, which are easily destroyed by toxic human serum, were derived from the subcutaneous tissue of a C_3H mouse, and were subsequently altered by treatment with a carcinogen (3,24). It is possible that their sensitivity to toxic human serum is related to their heterogenous origin (mouse), to their fibroblast-like nature, or to changes induced in the cells by the carcinogen. In an attempt to clarify the problem, serums which were toxic to strain L cells were also tested on five other cell strains. These included three strains of epithelium-like cells: HeLa (6, 25) derived from a human epidermoid carcinoma, Chang's kidney cell strain (1, 19) derived from normal human kidney, and Chang's conjunctiva cell strain (1, 2, 19) derived from normal human conjuctiva tissue; and, in addition, two strains of fibroblast-like cells: Pomerat's FF cell strain[b] derived from cartilage from the foot of a human fetus, and strain SK/C_3H derived from the subcutaneous tissue of a C_3H mouse. Strain SK/C_3H was

established in this laboratory. Two types of strain HeLa cells were used. One type was routinely grown in human serum, and the other had been adapted to grow in horse serum (22). All other cell strains used in the experiments to be described were grown routinely in medium containing horse serum.

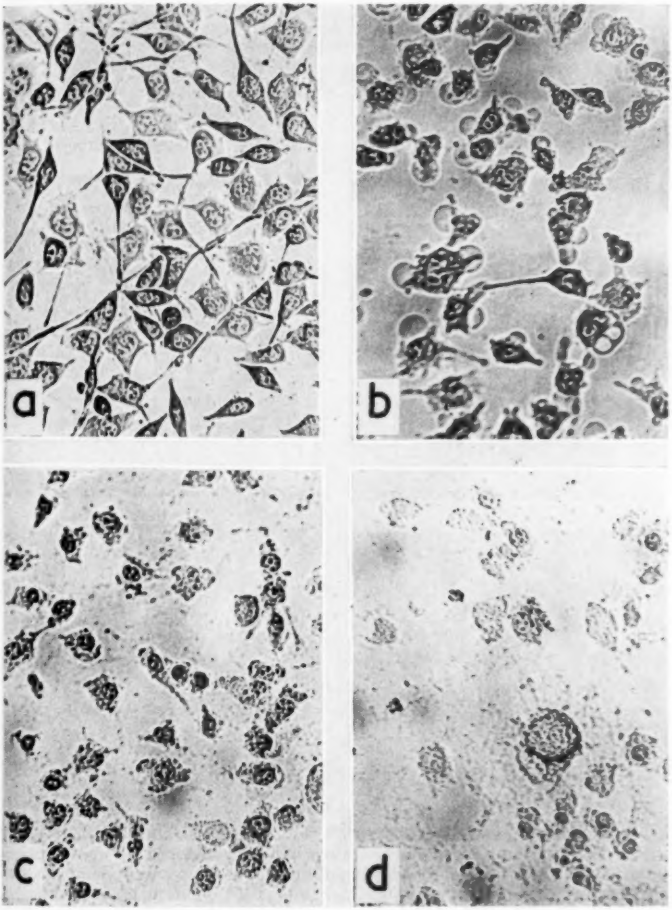

FIG. II. Reaction of strain L cells to toxic human serum: a. strain L cells before addition of toxic serum; b. 2 min. after addition of toxic human serum; c. 8 min. after addition of toxic human serum; d. 30 min. after addition of toxic human serum. Bright-field photographs of unstained cells fixed in Ringer-formaldehyde. (Magnification 400X).

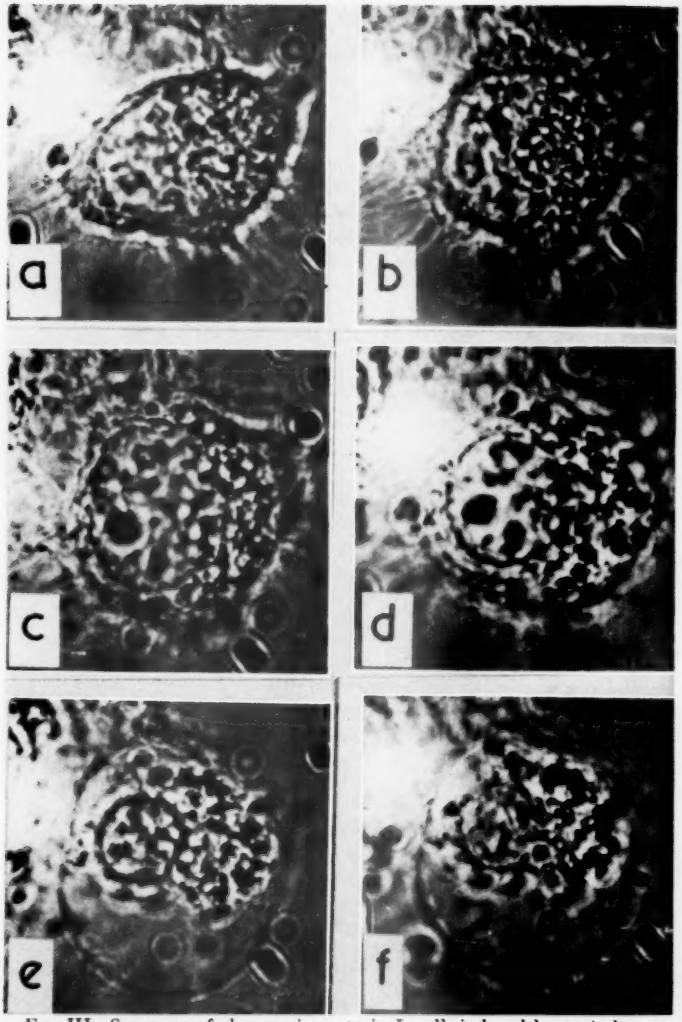

FIG. III. Sequence of changes in a strain L cell, induced by toxic human serum: a. strain L cell before addition of toxic human serum; b. 26 min. after addition of toxic human serum; c. 52 min. after addition of toxic human serum; d. 73 min. after addition of toxic human serum; e. 112 min. after addition of toxic human serum; f. 124 min. after addition of toxic human serum. Selected from a series or photographs taken every 2 minutes for 30 minutes before toxic serum was added, and every 2 minutes for 2 hours after addition of toxic serum. The culture was kept at 37° C. Bright field photographs of living, unstained cells. Magnification 1630X.

Serum which killed strain L cells had a similar toxic effect when incubated with strain FF and Sk/C_3H cells but only slight or no effect on the two types of strain HeLa cells, and on Chang's conjuctiva and Chang's kidney cells (Table 4).

TABLE 4

Toxicity of Human Blood Serum on Various Cell Strains Compared to Toxicity to Strain L Cells

Cell Strain	Origin of Cells	Appearance of Cells	No. of Serum Samples Tested	Degree of Serum Toxicity I To Cells Other Than Strain L Mean ± S.E.	II To Strain L Cells Mean ± S.E.	Ratio I/II	P
HeLa (grown in human serum)	human epidermoid carcinoma	epithelium-like	7	0	2.0±0.6	0	<0.01
HeLa (grown in horse serum)	human epidermoid carcinoma	epithelium-like	12	0.2±0.1	3.7±0.2	0.05	<0.01
Chang's kidney	human kidney	epithelium-like	14	0.6±0.1	2.9±0.3	0.22	<0.01
Chang's conjunctiva	human conjunctiva	epithelium-like	13	0.7±0.1	3.2±0.4	0.22	<0.01
SK/C_3H	subcutaneous tissue of C_3H mouse	fibroblast-like	22	2.1±0.2	3.2±0.1	0.66	>0.05
FF	fetal human cartilage	fibroblast-like	14	2.1±0.4	3.1±0.3	0.67	>0.05

In another series of experiments HeLa cells were grown together with L cells, and Chang's kidney cells with strain FF cells. When toxic serum was added to the mixed cell cultures, cells of strain L and FF were killed in every culture, whereas strain HeLa and Chang's kidney cells remained alive (Fig. IV). Twenty-four hours after the addition of the serum the epithelial cells were even observed in mitosis, and the number of mitoses increased during the following days.

5. *Interaction between toxic human serum and strain L cells.* The extent of destruction of strain L cells by human serum during three days of cultivation has been the basis for determining the degree of serum toxicity (4). Routinely, test cultures were examined once daily and, generally, the most severe effect on cell cultures was observed after the first 24 hours of culture with toxic human serum. Any subsequent increase in cell number depended on the severity of cell destruction during the first 24 hours. When very toxic serum was added to a culture, almost total cell destruction had occurred after the first 24 hours, and there was no increase in cell number during the following days. When, however, a less toxic serum was added, a smaller number of cells were killed during the first 24 hours and on continued incubation

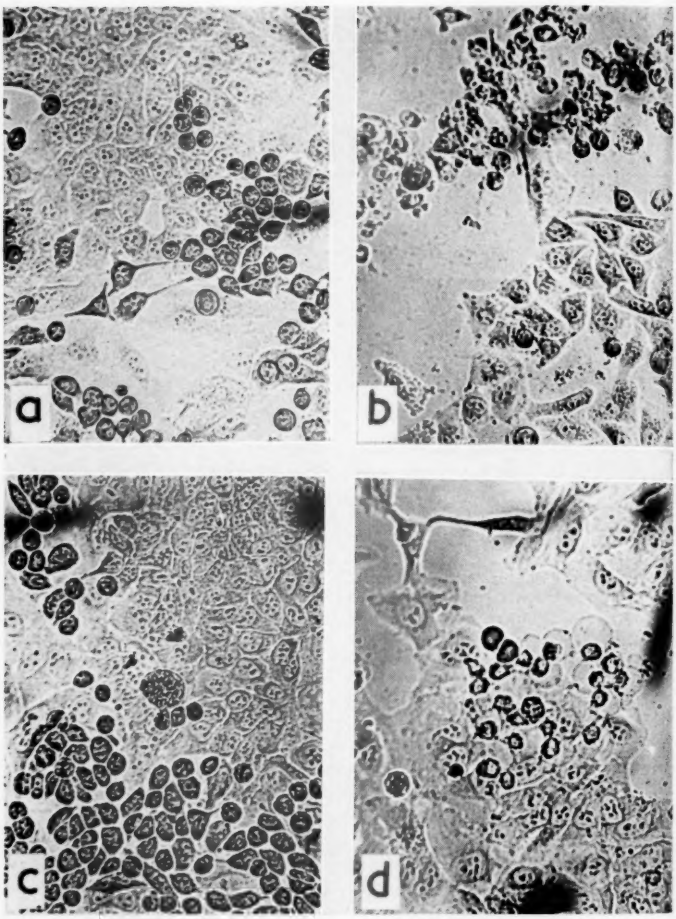

FIG. IV. Effect of toxic human serum on mixed cell cultures: a. strain HeLa and strain L cells before addition of toxic human serum; b. Strain HeLa and strain L cells 24 hours after addition of toxic human serum; c. Chang's kidney cell strain and strain FF cells before addition of toxic human serum; d. Chang's kidney cell strain and strain FF cells 24 hours after addition of toxic human serum. Bright field photographs of living cultures. (Magnification 400X.)

the surviving cells began to multiply (Fig. V). The serum had apparently been detoxified through the inactivation of toxic substances either by the cells or by the incubation temperature.

To investigate this problem, toxic serum was incubated with strain L cells, for 24 hours at 37° C., and was then removed from the cultures and added to new cultures of strain L cells. The serum was no longer toxic and could then support cell growth. Toxic serum incubated for 24 hours without cells was found to be slightly less toxic than before incubation (Fig. I), but it was not detoxified to the same extent as serum incubated with cells. Toxic serum was also detoxified when it was left in contact with strain L cells at room temperature or at 4° C. for 24 hours. An important factor in the detoxication was the presence of an excess of cells. Living cells were not necessary, and cells killed by boiling, or cells collected over a period of time and stored at —20° C., served equally well.

Detoxication of the serum by living cells was accompanied by reactions of the cells themselves. The reactions varied according to the temperature at which detoxication occurred. At room temperature and at 37° C. the serum was detoxified and the cells were killed. At 4° C. the serum was detoxified but the cells remained alive. Cells which were in contact with toxic serum for 24 or 48 hours at 4° C. could be grown at 37° C. if the toxic serum was replaced with a growth medium. If the toxic serum was not removed, and the cultures were changed from 4° C. directly to 37° C., the cells were killed within a few minutes.

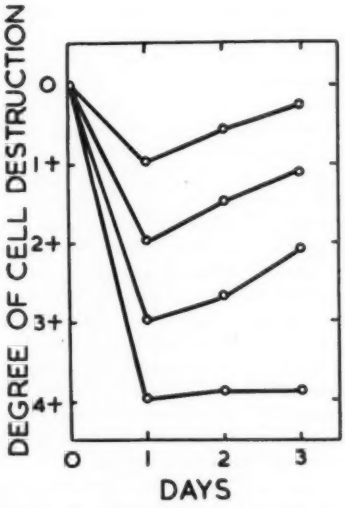

FIG. V. Reaction of strain L cell cultures to toxic human serums during a period of 3 days. Each point on the curves represents the average degree of cell destruction in 12 sets of 3 cultures each, caused by 12 different serums.

On incubating cells with toxic serum at 4° C. the serum was rendered non-toxic, but the cells remained alive; this suggested that at this temperature the cells were able to absorb the toxic substances. The following experiments were done to demonstrate whether or not the cells removed the toxic substance from the serum: Cultures of strain L cells were divided into two groups. Toxic serum was added to the cultures in group I, and non-toxic serum to those in group II. The two groups were kept at 4° C. for 24 hours. The serum was then removed from cultures in both groups, *non-toxic* human serum was added, and the cultures were incubated at 37° C. The cultures were examined microscopically every 10 minutes. Within the first 10 minutes the cells of the cultures of group I were dead, but the cells of the group II cultures were alive even after one hour of incubation (Table 5). The cells of the group I

TABLE 5

Effect of Heated and Unheated Non-toxic Serum on Strain L Cells Previously Exposed to Toxic and Non-toxic Serum at 4° C. for 24 Hours

Cultures of Strain L Cells Kept for 24 hr. at 4° C. with:	Degree of Cell Destruction After Addition and Incubation at 37° C. with: Non-toxic Serum	Heated Non-toxic Serum
Toxic serum (group I)	++++	+
Non-toxic serum (group II)	+	0

cultures had apparently absorbed toxic substances from the serum, but these could exert their toxic effect only at 37° C.

In a second experiment, cultures of strain L cells were again divided into two groups, toxic serum was added to group I, non-toxic serum to group II, and the cultures were kept at 4° C. for 24 hours. This time, however, after removal of the serum from the cultures in both groups, non-toxic serum, heated at 56° C. for 30 minutes, was added. No cells were killed in either group of cultures during the subsequent 24 hours of incubation (Table 5). Although the cells in the second experiment must have absorbed the toxic substance from the serum at 4° C. as shown by the first experiment, apparently something was lacking in the heated non-toxic serum to complete conditions for cell death in the group I cultures of the second experiment. This result points to the presence of a heat-labile substance which by itself is non-toxic, but which with cells previously exposed to toxic serum will bring about cell death.

To demonstrate the presence in toxic human serum of two substances which are necessary to kill strain L cells, the following experiment was done: Toxic serum which had been heated at 56° C. for 30 minutes was added to a series of strain L cell cultures. The cultures were divided into groups and to every group except one, non-toxic human serum was added in different concentrations. As shown in table 6, heat detoxified the serum. The addition of *non-toxic* serum to heat-detoxified serum restored the toxicity (Table 6). This experiment was repeated with 5 different serums with similar results. It seems that in toxic human serum there are at least two substances, one heat-stable and the other heat-labile. The cells are killed only when both substances

TABLE 6

Restoration of Toxicity by Addition of Non-toxic Serum to Serum Detoxified by Heat

No. of Cultures	Amount of Toxic Serum (ml.)	Amount of Heated Toxic Serum (ml.)	Amount of BSS (ml.)	Amount of Non-toxic Serum (Drops)	Average Degree of Toxicity After 3 Days at 37° C.
3	0.5	..	0.5	..	++++
3	..	0.5	0.5	..	0
3	..	0.5	0.5	2	0
3	..	0.5	0.5	4	+
3	..	0.5	0.5	8	++
3	..	0.5	0.5	12	++++
3	..	..	1.0	12	0

are present in proper concentration and at a suitable temperature. This type of reaction is somewhat similar to an antibody-antigen reaction with complement fixation. To determine whether the heat-labile substance is actually complement, human serum which had been detoxified by heat was added to series of strain L cell cultures. The cultures were divided into groups, and to each group except two, guinea pig serum was added in increasing quantities. To one of the groups to which the guinea pig serum had not been added, non-toxic human serum was added. All cultures were incubated at 37° C. for 24 hours and then examined microscopically. The addition of guinea pig serum did not restore the toxicity of the heated serum, in contrast to the addition of non-toxic human serum (Table 7). This experiment indicates that the

TABLE 7

Effect of Addition of Guinea-pig Serum to Human Serum Detoxified by Heat

No. of Cultures	Toxic Human Serum (ml.)	Heated Toxic Human Serum (ml.)	Hanks' Balanced Salt Solution (ml.)	Guinea Pig Serum (Drops)	Non-Toxic Human Serum (Drops)	Average Degree of Toxicity After 24 hrs. at 37° C.
3	0.5	..	0.5	..	..	++
3	..	0.5	0.5	..	..	0
3	..	0.5	0.5	2	..	0
3	..	0.5	0.5	4	..	0
3	..	0.5	0.5	6	..	0
3	..	0.5	0.5	..	10	+
3	..	..	1.0	..	10	0

heat-labile substance in human serum is not identical with the complement of guinea pig serum. In a similar experiment, horse serum was substituted for guinea pig serum with the same result.

To determine whether the heat-labile substance is identical with the complement of human serum, another experiment was devised. It was based on the supposition that if the heat-labile substance is human serum complement,

then there should be a difference in its titer in toxic serum, before and after exposure of the serum to strain L cells at 37° C. Toxic serum was therefore added to cultures of strain L cells incubated at 37° C. A control sample of the same serum, but without cells, was also incubated at the same temperature and for the same time. When complete cell destruction had taken place (after 30 minutes of incubation) the cultures were centrifuged, and the serum was removed. The serum which had been incubated with strain L cells, and the control sample of serum were both titrated for complement with sensitized sheep red blood cells. The control serum contained 1 minimal hemolytic dose (M.H.D.) in 0.1 cc. of serum. The serum which was incubated with the cells contained one minimal hemolytic dose (M.H.D.) in 0.2 cc. The difference is not significant.

Discussion

The toxicity of some human serum to strain L cells has been shown to be due to the presence in the serum of a heat-labile substance and a heat-stable substance. When toxic serum was dialysed against balanced salt solution the serum was not detoxified, which indicates that the toxic substances are large molecules or are bound to large molecules. Strain L cells were killed by these substances only when both were present in proper concentrations and at certain temperatures. The heat-labile substance could be inactivated by heating the serum at 56°C. for 30 minutes and the heat-stable substance could be absorbed from the serum by living or boiled strain L cells. This suggests that there are heat-stable substances in the serum.

Toxic human serum which was detoxified by heating could be re-activated by the addition of non-toxic human serum. This indicates that the heat-labile substance is present in non-toxic serum. Addition of guinea pig or horse serum did not re-activate heat-detoxified human serum, suggesting that the heat-labile substance is not identical with the complement of these serums. Attempts to demonstrate its identity with human complement failed, although the procedures may not have been sensitive enough.

The experiments dealing with the sensitivity of strain L cells to toxic human serum have shown that the sensitivity of the cells is not due to the fact that cells originated from another species than the serum, the proof being that FF cells were also killed by the toxic serum. Nor is the sensitivity related to the treatment of strain L cells with a carcinogen, because the SK/C_3H cells were also killed by the toxic human serum. These cells were de-

rived, as were strain L cells, from subcutaneous tissue of a young male C_3H mouse, but had never been exposed to a carcinogen. Sensitivity of cells in tissue cultures to toxic human serum appears to be related to the nature of the cells, since human fibroblast-like cells are killed by toxic human serum, whereas human epithelium-like cells are not (Table 4). The difference in sensitivity between fibroblast-like and epithelium-like cells is probably not due to a different origin from the three germ layers. In mixed cultures of cells of Chang's kidney strain and Pomerat's FF strain, only the FF cells were killed, although the ancestors of both cell strains are of mesodermal origin. Factors responsible for the different sensitivity of these strains to toxic human serum must therefore have been introduced during their differentiation in the postgastrula stage. Puck, Cieciura and Fischer (23) noticed that fibroblast-like cells, in general, are much more sensitive to their environment than epithelium-like cells. The differences in sensitivity of cells to toxic human serum might be utilized in isolating epithelium-like cell strains from mixed cell cultures, a procedure which generally meets with great difficulties.

The morphological changes observed in strain L cells exposed to toxic human serum are similar to changes observed in tissue cultures of embryonic and adult, and of normal and malignant cells, after exposure to specifically produced antiserums (9, 10, 12, 13, 14, 16, 18, 20, 21). In each of these antiserums different antibodies were present, but all of them produced similar morphological changes in the cells, indicating that they all attack and injure cells in a similar way. Latta proposed that a consequence of the reaction of antibodies and complement with surface antigens of cells is a change in the permeability of the cell membrane (14). The immediate appearance of blebs, observed after the addition of toxic serum to strain L cells, indicates that the cell membranes are affected. This bleb formation is different from that produced by the addition of Victoria Blue to cultures. The bleb formation caused by toxic serum is unaffected by the addition of ATP, but the bleb formation caused by Victoria Blue is inhibited. An observation which emphasizes the importance of the area of the cell membrane exposed to the toxic serum, is that after the addition of toxic serum to very densely populated cultures of strain L cells, the cells tend to clump, and the cells

within the clumps are apparently unaffected by the serum. In a few days new growth originates from the clumps of cells (4). The resistance of epithelium-like cells to toxic human serum may be related to the protection of large portions of their cell membranes by the close approximation to neighboring cells, although this possibility seems unlikely.

The most important question, *i.e.*, the cause of the presence of the toxic substances in some human serum, remains unanswered. These substances may be acquired or inherited. If acquired, they may result from pathological or immunological processes. An attempt to find a higher titer of Forssman type antibodies in the serum of schizophrenic patients than in the serum of normal people failed (11). The presence of toxic substances in the blood serum of various animals has been reported previously. Lumsden and Kohn-Speyer (17) have observed in adult rabbit serum substances toxic to mouse cells which they called "heterotoxins." They were unable to distinguish the effect of heterotoxins from the effect of especially prepared cytotoxic antibodies against mouse cells. They also observed that chicken, sheep, goat and human serums were toxic to normal and malignant mouse cells. Lately, Grand (7) reported that the blood serum of normal adult rats was toxic to cultures of cells from a transplanted squamous cell carcinoma of the mouse but not to cultures of cells from normal squamous cell epithelium or from transplantable carcinoma of mice. Schrek and Preston (26, 27) observed that serum taken from rats in which Bagg transplantable lymphosarcoma had regressed was toxic to the tumor cells *in vitro*, but was not toxic to cells from the thymus of a normal rat. The patterns of cell destruction described by Lumsden and Kohn-Speyer (17), Grand (7), and Schrek and Preston (16, 17), are very similar to these observed when toxic human serum acts on fibroblast-like cells.

There are some similarities between the present investigation and that of Sjöwall (28). The frequency distribution of toxicity of serum from schrizophrenic patients to strain L cells in tissue culture (5) agrees well with that determined by Sjöwall by injecting mice with schizophrenic serum. Sjöwall suggested that the death of mice injected with serum was due to hemolysis, because hemoglobinuria was produced. If the serum was heated at 56° C. for 30 minutes before injection, the animals survived

the injection, there was no hemoglobinuria, and the serum lost its hemolytic properties *in vitro*. However, Sjöwall was unable to explain why homoglobinuria was absent, because he thought that mouse serum should have enough complement to reactivate the hemolytic properties of heated schizophrenic serum. I also failed to restore the toxicity of serum by adding heterologous (guinea pig and horse) serum to heat-inactivated human serum.

In spite of the striking similarity of the effect of toxic human serum on fibroblast-like cells in tissue cultures to the cytotoxic reaction produced by antiserums, there is at present no conclusive evidence to prove that the toxic substances in human serum are immunologically produced, or are immunologically active.

Summary

1. The toxicity to strain L cells of some human blood serums, especially those from schizophrenic patients, has been shown to be due to the presence in the serum of a heat-stable and a heat-labile substance.

2. The toxic substances are non-dialyzable.

3. Strain L cells were killed when both substances were present in the serums in proper concentration and at proper temperature.

4. The heat-labile substance was inactivated by heating the serum at 56° C. for 30 minutes and the heat-stable substance was absorbed by either living or dead strain L cells.

5. The toxicity of toxic human serum which had been inactivated by heat was restored by the addition of non-toxic human serum. The addition of guinea pig or horse serum did not reactivate the toxicity of the inactivated toxic serum.

6. Toxic human serum killed strain L cells, Pomerat's strain FF cells, and SK/C_3H cells, but did not kill strain HeLa cells, Chang's kidney cells, or Chang's conjuctiva cells.

7. Morphological changes induced in strain L cells by toxic human serum are described.

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[b] This cell strain, as yet not described, was established in November 1956 by Dr. C. M. Pomerat and associates. The strain was obtained from Dr. T. C. Hsu of the M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

THE DEVELOPMENT OF A HUMAN AMNION STRAIN OF CELLS[a]

MARIO V. FERNANDES[b]

Current interest in the cultivation of cells from human amniotic membranes in tissue culture was greatly stimulated by the work of Zitcer, Fogh and Dunnebacke (1955). Further studies in various laboratories have shown amnion cultures to be suitable for investigations of many viruses (Fogh and Lund, 1955; Dunnebacke, 1956; Wilt, Stanfield and Leindl, 1956; Weinstein, Alexander, Yoshihara and Kirby, 1956; Lahelle, 1957). Since cells from the primary outgrowth do not seem to be essential for virus propagation and because the use of strains offers several practical advantages, particularly the elimination of a long trypsinization procedure and difficulties involved in collecting material in good condition for tissue cultures, several workers succeeded in developing amnion cells in continuous culture. The first successful effort (FL strain) was reported by Fogh and Lund (1957) from the Virus Laboratory of the University of California at Berkeley.

The present report is concerned with a line (amnion strain-Fernandes) of epithelial-like cells derived from a normal human amniotic membrane, established in a short period by trypsinization of the primary tissue and using a medium without embryonic extract.

Materials and Methods

Placental tissue from a healthy woman was obtained from the John Sealy Hospital in Galveston, Texas. It was handled aseptically in the delivery room and collected in a sterile pan.

Human, horse and calf sera were inactivated for 30 minutes at 56° C. and kept at 4° C. Trypsin solution (Difco 1:250) was used at 0.25 per cent in Gey's balanced salt solution (BSS) without calcium and magnesium, sterilized by Seitz filtration and kept frozen at —20° C. The pH was adjusted to 7.8 by the addition of N/10 sodium hydroxide after sterilization. Eagle's medium was used with 10 per cent horse serum for establishing and maintaining the strain. In addition, Medium No. 199 with 20 per cent horse serum and an ascitic fluid nutrient consisting of 50 per cent ascitic fluid, 45 per cent Gey's BSS and 5 per cent embryonic extract were tested. All media contained 100 units of penicillin and 100 gamma of neomycin per millilitre.

Enumeration of cells was made after they were washed and resuspended in a known volume of medium. They were counted with the use of a hemocytometer in the usual way.

Experimental Observations and Results

Procedure. Fetal membranes were brought to the tissue culture laboratory within one hour. The amniotic layer was quickly stripped off, dissected and cut into fragments approximately 2 mm. square. These were washed several times with Gey's BSS. The fragments were then cut into very small pieces with the use of iridectomy scissors. They were washed three times in Gey's BSS containing 1000 units of penicillin and 1000 gamma of neomycin per millilitre, and placed in a 250 ml. Erlenmeyer flask. For digestion 30 ml. of 0.25 per cent trypsin in Gey's BSS, which had been pre-heated to 37° C., were added to the flask containing the amnion fragments together with a small magnetized neutral glass-coated iron cylinder. The flask was then placed on a magnetic mixer and the fragments were stirred at a slow rate for a period of 25 minutes. After removal from the mixer the flask was allowed to stand for about one minute before the supernatant fluid was removed and discarded. Another 30 ml. of 0.25 per cent trypsin were added and the same procedure was repeated including discarding of the supernatant. The fluid from four subsequent trypsinization procedures was collected in 40 ml. centrifuge tubes and placed in the refrigerator until the entire process was completed. The cells were then centrifuged at 1000 rpm for 15 minutes at approximately 4° C. The trypsin solution was decanted and discarded. The cells were washed twice in Eagle's medium, centrifuged again for 15 minutes and finally resuspended in Eagle's medium. The cells were counted with a hemocytometer and dispensed in roller tubes (100,000 cells in one ml. per tube), Rose chambers (100,000 cells) and T30 flasks (800,000 cells).

Cultivation of the Cell Strain. The cells from which this strain was developed originated from one of the T30 flasks. They were all of the same type—large, clear, epithelial-like elements with little or no granularity. The nucleus was round, clear and centrally located. Two, three or four nucleoli were seen in each nucleus. At the end of the 10th day the flask was completely covered by a sheet of flat epithelial-like cells, as shown in figure 1. At this time many cells were observed to be in mitosis indicating a high rate of division.

The first change of medium was 48 hours after putting the cell suspension in the T30 flask in order to eliminate floating debris. Subsequently the medium was changed every five days, and after the 15th day it was replenished every day. On the 18th day the first subculture was made. Trypsin was not used. A rubber policeman was employed to scrape the cells from the glass. These were dispersed with a 30 ml. syringe fitted with a No. 15 needle and a final suspension of 100,000 cells/ml. was prepared. This suspension was distributed into two T30 flasks. Good growth of cells was obtained and 24 hours later they were well attached to the glass. They now appeared to form a clear sheet. The cells began to multiply very rapidly on the second day showing a very high percentage of mitotic figures. The medium was renewed

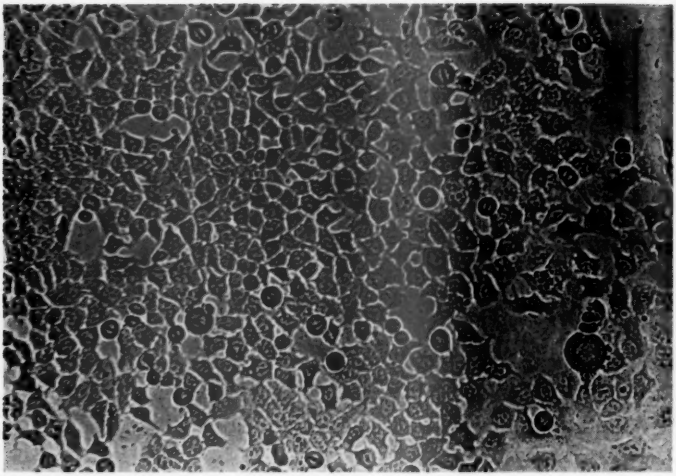


FIG. 1. Amnion strain (Fernandes). Living culture in a T30 flask. Primary culture after 10 days *in vitro*. Note the high frequency of mitotic figures. Ordinary light microscopy. The scale of magnification is shown in the upper right area of the figure.

daily due to the very rapid cellular multiplication. On the 5th day the entire surface of the flask was covered. There was no apparent morphological change, therefore a second subculture was made. Using the same technique as described, the cells were then transferred in two T30 flasks and six Rose chambers. The cells in two of the Rose chambers were fixed on the 12th day of incubation and stained with Jacobson's method, using Gey's BSS made hypotonic by diluting one part in 9 of tap water in order to facilitate the counting of the chromosomes. Dr. Yuh Nakanishi of this laboratory found that the range of chromosome numbers was from 60 to 77 and that the modal value was 67.

In the T30 flasks the cells continued to multiply very rapidly necessitating subculture on the 8th day. After this, subcultures were made every five days and at the present time they are in the 44th passage, which is about seven months from the date of the original trypsinization.

Growth of the Amnion Strain (Fernandes) in Various Media. During the entire course of study some of these cells have been grown in Eagle's medium with 10 per cent horse serum. The growth curves which were prepared for the 10th, 26th and 27th subcultures are shown in figure 2. The technique for studies of cell multiplication was the same as that described by Fogh and Lund (1957) with a few modifications. Using hemocytometer

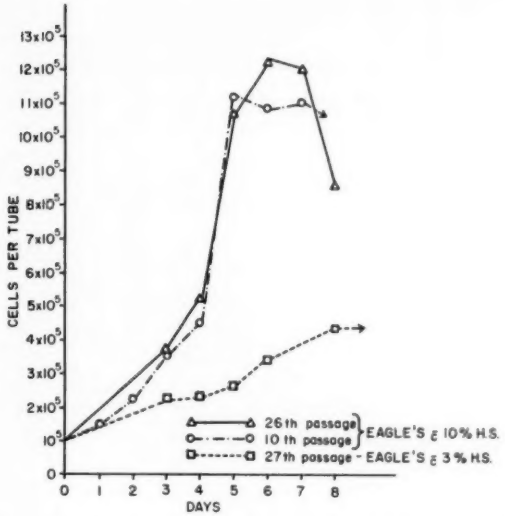


FIG. 2. Growth curves for amnion strain (Fernandes) in relation to culture generation, composition of media and transfer day.

technique, 100,000 cells/ml. were dispensed in test tubes (1 ml. per tube). Tubes were incubated at 37° C. At 24-hour intervals during the next 8 days, 7 tubes were counted each day. A rubber policeman was used to remove the cells completely from the glass surface and the average number of cells was calculated with the use of the hemocytometer method. Two counts were made for each tube. The value for each day was the average of the 7 tubes.

By the second day the cells were approximately twice as numerous as in the original inoculum. Beginning at this time the cells multiplied very rapidly and on the 5th day the number was approximately 1,200,000. At this time the proliferation index, which is defined as the number of cells for each day divided by the number of cells inoculated, was 11.67. This index is represented in figure 3 in relation to the FL strain (*cf.*, Fogh and Lund, 1957). The mitotic rate during the 4th and 5th days was approximately 10 per cent.

After the 5th day the number of cells began to decrease, perhaps due to an inadequate volume of medium in relation to such a high multiplication rate. At the end of the 7th day, the pH of

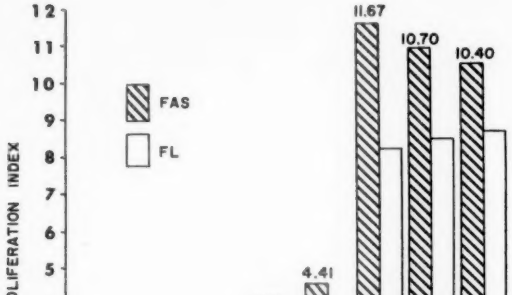


FIG. 3. Proliferation index (see text) for the amnion strain (Fernandes) is shown in the bars with oblique lines (FAS) with exact values for each day in comparison with results obtained by Fogh and Lund (1957) represented by clear bars.

the tubes was approximately 6.9. When using only 3 per cent horse serum, the increase in the number of cells was reduced as can be seen in figure 2. This situation would seem to be particularly useful in the study of certain viruses.

The increase in cell concentration in time was studied using several types of media at various percentages and species of sera. The pertinent data which were obtained are shown in figure 4. The morphology of the cells remained constant. This was also found when the medium contained only 3 per cent horse or calf serum. Only when ascitic fluid was used in the composition of the fluid nutrient did the cells present some structural variation. Under these conditions they appeared somewhat more separated and some assumed the fibroblast-like form.

Under standard conditions in which Eagle's medium containing 10 per cent horse serum, cells were regularly found to become quickly attached to the glass substrate. Selected frames from a film record of typical results obtained in a Rose chamber

photographed at one frame per minute from a 13th passage culture are shown in figures 5–12.

The appearance of cells in two-day cultures from the 44th transfer generation as revealed with dark phase microscopy are represented in figures 13–16. The frequency of mitotic figures and the presence of multiple nucleoli are illustrated in figure 13. Irregularities in the plasma membrane of the type which is characteristic of ectoplasmic activity as seen in cine records are shown in figure 14. The mitochondrial pattern in the juxtanuclear zone is demonstrated in uninucleate cells (Fig. 15) and in a binuclear element (Fig. 16).

Susceptibility of the Amnion Strain (Fernandes) to Viral Infection. Cytopathogenic changes resulted from the inoculation of many viruses. Figure 17 summarizes the data obtained on the susceptibility of these cells to the viruses studied thus far. The changes attributable to the bluetongue virus consisted primarily of the shrinking and rounding up of cells, increased granularity, and the production of optically dense nuclei. As the injury increased, holes appeared in the epithelial sheet and the cells sep-

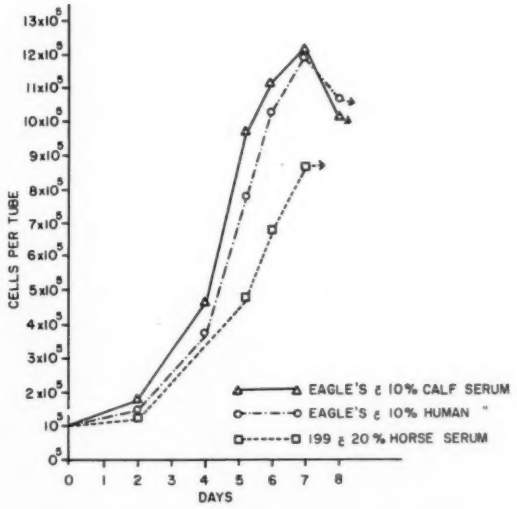


FIG. 4. Growth curves for amnion strain (Fernandes) in relation to the composition of the medium tested at the 40th passage.

arated from one another. Finally, the cells rounded up, became detached from the glass and floated in the medium. A detailed report on these observations is in preparation.

Discussion

A human amnion cell line was developed by trypsinization of the primary tissue and cultivated in a medium without embryo extract. The appearance of cells was epithelial-like at all stages. The mixed population of large and small cells described by Zitcer, Fogh and Dunnebacke (1955) was not observed. They did not grow out of predominantly fibroblastic cultures as described by Jordan (1956), Drew (1957) and Westwood and coworkers (1957). Moreover, no "slow" period of multiplication, as noted by these investigators and by Fogh and Lund (1957), was encountered in the development of this strain.

Since the chromosome count made on the 35th day after the original trypsinization indicated a subtriploid number (the modal value was 67) and also because the multiplicative rate of the cells was very high at this time, we assume that the strain was established before the 35th day.

The strain is now in its 44th passage which is about seven months after the initial trypsinization. It appears to be easily maintained in a variety of nutrient media, it has a high multiplicative rate and can provide large amounts of cells for viral diagnosis, vaccine production and other research purposes.

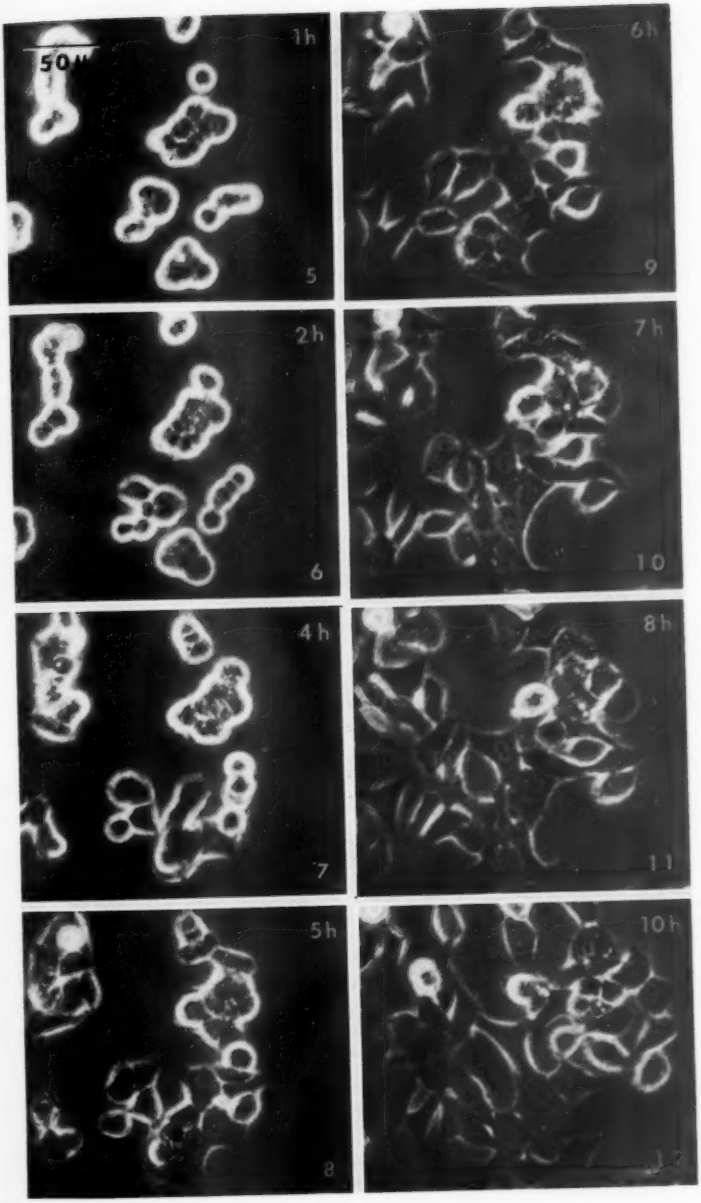
Summary

A strain of epithelial-like cells, derived from a normal human amniotic membrane by trypsinization, is described.

The appearance of the cells was epithelial-like at all stages of cultivation and the multiplicative rate also remained very high.

The strain was assumed to have been established by the 35th day on the basis of chromosome counts and the rate of cell division.

FIGS. 5–12. Establishment of cells from a 13th passage culture of the Fernandes amnion strain in a Rose chamber containing Eagle's medium with 10 per cent horse serum. Selected cine film frames show the attachment of the cells to the glass. The scale for the magnification is shown in the upper left area of figure 5 and the time intervals are given in the upper right areas of each figure.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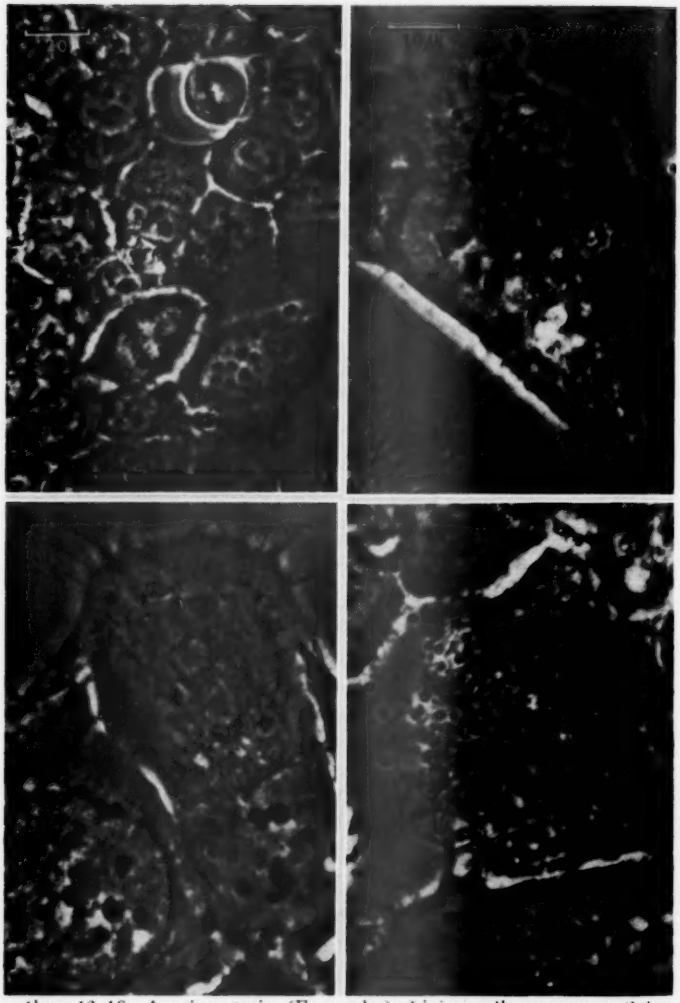

FIGS. 13–16. Amnion strain (Fernandes). Living cells as seen in 2-day cultures from the 44th transfer generation. Note the presence of several mitotic figures and multiple nucleoli of cells in figure 13. Figure 14 shows irregularities of the plasma membrane, while figures 15 and 16 demonstrate the typical arrangement of organoids.

Dark phase microscopy.

The scale of magnification for figure 13 is shown in the upper left area of that illustration. The scale for figures 14–16 is shown on figure 14.

Bluetongue virus	4+	Degeneration of almost all cells
Adenovirus* Type 1	4+	"
2	4+	"
3	4+	"
4	4+	"
6	4+	"
7	4+	"
Newcastle disease* (Victoria strain)	4+	"
Mumps (ABC strain)*	4+	"
Poliovirus** Type I	4+	"
II	4+	"
III	4+	"
Herpes simplex**	4+	Multinucleated cells. Intranuclear inclusions
Measles**	2+	Degeneration and intranuclear inclusions

* Kindly supplied by Dr. Leonard Hayflick, Department of Microbiology, University of Texas, Galveston.

** Kindly supplied by Dr. Morris Pollard, Department of Public Health & Preventive Medicine, University of Texas, Galveston.

FIG. 17. Susceptibility of amnion strain (Fernandes) to viral ingestion as reflected by cytopathogenic changes.

Growth characteristics in various media with several species of sera and concentrations are reported. The potential value of this strain for virological work is suggested by preliminary tests.

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ON THE ROLE OF PHAGES IN SEROTYPIC VARIATION OF C. DIPHTHERIAE

LUDWIK FLECK[a] AND WITOLD KOZAK

The antigenic changes of bacteria have been the subject of numerous investigations in connection with phage action. D'Herelle (1938) observed the increased or decreased agglutinability of resistant bacterial strains as compared with original strains. Zinder and Lederberg (1952) published the theory of genetic transduction whereby the filtrate of a phage containing culture is able to transfer a genetic material from this strain to another, leading to permanent acquisition by the recipient strain of a new character from the first donor strain. The mentioned authors succeeded in transduction of the "i" antigen from S. *typhimurium* to S. *typhi*; Kauffmann performed the transduction of "b" and "i" antigens from S. *typhimurium* to S. *paratyphi B* etc.

Hewitt (1952) suggested the idea of phage autoadaptation as a mechanism of type evolution in C. *diphtheriae*. The phage "can be adapted to lyse the parent bacterial strain leaving intact only the resistant mutants which in turn can be attacked by newly adapted virus particles. The evolution of the bacterial strain and the virus proceed side by side, one controlling the other."

In the course of our investigations on the serologic differentiation of C. *diphtheriae*, we found a surprisingly large number of serotypes. We have differentiated, in Poland, 14 types, but are still discovering new ones. Some of them are very rare, occurring only once or twice or in a few of our 2,000 strains. During a diphtheria epidemic lasting several years (1953–1957) we found in a certain region the sudden appearance of a larger number (21) of corynebacteria strains belonging to serotype 49 previously absent in this region for at least 2 years. Besides normally agglutinating strains, we found some (16) strains weakly agglutinated by the standard serum anti–49 (up to 12–25 per cent of the titer), and reacting with no other standard serum. Yet in the year 1957 (January to July), no strains either nor-

mally or weakly agglutinating with the 49 serum strains were found again in this region. The simultaneous appearance of normally and weakly agglutinating strains seems to demand a joint explanation. An assumption seems to be acceptable that they arose in the course of a typogenic process in which the weakly agglutinating strains may represent a transitional stage.

The large dissemination of phages among corynebacteria attracts some attention. We succeeded in phage release by UV-irradiation from 37 strains out of 102 investigated. By using other additional methods the percentage of lysogenic strains found would probably rise still more. The adaptation of phages from one serotype to another is, under laboratory conditions, not too difficult and, of course, possible in nature. We found that carriers of several different corynebacteria strains in the throat and nose are not rare. We found in some children several (up to 7) different strains, toxic and atoxic.

In such an environment, favorable conditions are offered for adaptations from one strain to another, and for evoking a variability of types which may possibly cause a change in the course of the epidemic.

There is evidence of a certain role played by the antibacterial (type specific and species specific) immunity in diphtheria. A change in the serological character of the bacilli may disturb the equilibrium in the immunological situation of an area.

Thus the problem of the role played by phages in the variation of serotypes of C. *diphtheriae* becomes not only theoretically interesting, but also practically important. We tried to contribute to the solution of this problem by means of laboratory experiments.

Materials and Methods

The investigations were made on five C. *diphtheriae* strains: *14*, *24*, *29*, *32* and *1174* (Freeman). The first four of these originated from patients or carriers and belong to serotype *24* of our nomenclature (gravis, toxigenic). The fifth strain originates from the Washington collection and belongs to serotype *25* (mitis, nontoxigenic).

Types *24* and *25* have a deep-seated thermostable antigen O. The anti-*24* serum agglutinates the *25* strain up to 3–25 per cent of its titer. This agglutination disappears when we use an

anti-*24* serum absorbed with homologous boiled strain. The anti-*25* serum, however, does not agglutinate the strains *24*, the O antigen being less accessible because it is covered by K antigen.

The phages used for the experiments were derived from the lysogenic strains *14* and *24* on the indicator strains *29* and *32*. They were numbered *14/29*, *14/32*, *24/29*. The induction was performed according to Fisk (1942) or Lwoff, Simonowitch, and Kieldegaard (1950) by means of UV-irradiation. The cultures were sterilized with chloroform according to Fredericq (1952).

Phage *14/29* originally did not act on the heterotypic strain C. *diphtheriae 1174*. The adaptation was performed by means of a multiple common agar culture of strain *29* and *1174* with several drops of phage *14/29*. Already after three passages, there appeared single plaques out of which we got the phage *14/29/1174* after three-fold multiplication on the strain C. *diphtheriae 1174*, according to Adams. This phage, contrary to the original phage *14/29*, did not lyse C. *diphtheriae 29*, but lysed *1174*.

The resistant mutants were derived in the usual way. From the colonies resistant and lysogenic on the original strain as an indicator, the following variants were chosen at random: M1, M2, M3 derived from the strain *32* by the action of phage *14/32*. N1, N2, N3, derived from strain *29* by the action of phage *24/29*. P1, P2, from strain *29* by the successive action of phages *24/29* and *14/32*. R1, R2, R3, R4, R5 from the strain *1174* by the action of the heterotypic phage 14/29/1174. These 13 variants were serologically compared with the original strains, using the standard sera, anti-*24* (titer 1:1000) and anti-*25* (titer 1:4000).

Results

The strains M1, M2, M3 and N1, N2, N3 reacted serologically in the same way as the original strains.

The strains P1 and P2 agglutinated with the same anti-*24* serum much less than the original strain *29*. P1 was agglutinated only up to 1:256, P2 only to 1:128, whereas the original strain reached 1:1024. The serum anti-*25* did not agglutinate these strains, nor did it agglutinate the original strain. Thus the serological change evoked by the successive action of two homotypic phages was apparent. It consisted of a decrease in agglutinability to 12.5 and 25 per cent of the titer, respectively.

The strain R1 showed no significant serological changes as compared with the original strain *1174*. The strains R2, R3, R4 and R5 definitely changed their serological behavior. They ceased on the whole to be agglutinated by the serum anti-*24*, whereas the original strain *1174* was agglutinated up to 1:128 by this heterotypic serum. With the serum anti-*25*, R2 showed the same reaction as the original strain, R3. R4 diminished in agglutinability slightly. R5, however, was agglutinated up to 6.25 per cent of the original titer, 1:2000 (Table 1).

Thus we observed significant serological changes of several resistant strains, especially after their adaptation to heterotypic phage, and particularly the cross agglutination with common O antigen.

The described experiments were repeated 6 times, by using several other sets of strains and phages. We obtained almost identical results.

Twice we obtained resistant strains (variant R40) which entirely failed to agglutinate with the serum for the original strain. All the resistant strains were lysogenic. One resistant variant, R45, agglutinated with the heterotypic serum in a greater dilution than the original strain.

Some of the variants, such as R5 and R40, presented themselves as new serotypes.

In order to prove whether there is a correlation between the observed serological changes and the phage resistance, we made a number of passages of R1, R2, R3, R4 and R5 during two months on the Loefler medium. We succeeded in obtaining once more the sensitive variants. They showed in almost all cases restored agglutinability. In one case however the restoration led only to 25 per cent of the original titer.

Discussion

To explain the observed facts, it seems useful to make a hypothesis of agglutination inhibition due to an alteration of cell surface in resistant strains. This surface alteration disappears with the loss of resistance accompanied simultaneously with the reappearance of agglutinability. In this matter there could be a certain analogy to the W-V variation (F. Kauffmann) which gives rise to V form with the antigen Vi on the surface causing

TABLE 1

Agglutination of Resistant Strains R1, R2, R3, R4, R5, Derived From the Sensitive Strain 1174 by the Action of the Adapted Heterotypic Phage 14/29/1174

Strains		Dilution of the Standard Sera													
Original	Resistant	1:16	1:32	anti-*24* 1:64	1:128	1:256	1:16	1:32	1:64	1:128	anti-*25* 1:256	1:512	1:1024	1:2048	1:4096
1174		+++	+++	++	+	—	+++	+++	+++	+++	+++	+++	+++	+++	—
	R1	+++	++	—	—	—	+++	+++	+++	+++	++	++	++	+	—
	R2	+	—	—	—	—	+++	+++	+++	+++	+++	+++	++	++	—
	R3	—	—	—	—	—	+++	+++	+++	+++	+++	++	++	—	—
	R4	+—	—	—	—	—	+++	+++	+++	+++	+++	+++	++	+	—
	R5	—	—	—	—	—	+++	+++	+++	+++	+	—	—	—	—

the O-agglutinability. A reversion into W form connected with the loss of antigen Vi restores the O-agglutinability. The Vi antigen of the Salmonella, like the L antigen (Kauffmann) of Escherichia acts as an O-agglutination inhibitor.

In order to verify or to reject this hypothesis, we tried first to prove whether or not the resistant variants which do not agglutinate either with homologous or with heterologous sera, have lost their corresponding antigens. We found by absorption experiments that the resistant variant R40 obtained from C. *diphtheriae 1174* by the action of phage 1174, though not agglutinated by sera anti-*24* and anti-*25* easily absorbed from both sera all the agglutinins for the original strain *1174* when used as a suspension of cells disintegrated by repeated freezing and thawing. Thus, in our case, agglutinability change caused by phage action does not consist in a disappearance of certain antigens.

In order to prove whether or not the observed agglutination change is due to the appearance of a new outer layer of an antigenic substance, we immunized rabbits with the inagglutinable variant R40. The serum obtained showed after repeated absorption with the original strain *1174* no precipitating antibodies against the variant R40. Consequently, this variant contains no additional antigens, which could cover the original antigens of strain *1174*. Thus we found neither an appearance of a new antigen nor a disappearance of a primarily present antigen. It is nevertheless possible that the mechanism of the non-agglutinability after phage action is due to some other factor, *e.g.*, the presence of a non antigenic covering substance.

The loss in agglutinability in our case may result from mere structural changes, which mask or inhibit the reaction. The less specific cross agglutination with the deeper O antigen disappears more often than the more specific K agglutination, the antigen of which lies nearer the cell surface. Therefore, it would suffice to assume that the observed changes depend on an increase of the distance between the corresponding antigens and the cell surface, causing an inaccessibility of the antigens. Such changes would lead to a state described by Coombs in connection with the "agglutination of bovine red cells previously classified as inagglutinable by the building up of an antiglobulin-globulin lattice on the sensitized cells."

Whatever the mechanism of the investigated nonagglutinabil-

ity may be, we have to consider whether this is a way leading to an evolution of new serotypes. The observed serologic changes consist neither in loss of certain antigens nor in gain of new ones. Therefore, when we define a serotype as a collection of strains with a fixed set of certain partial antigens, we scarcely could look upon the described changes as a typogenic process. They could only mask the true antigenic structure of a strain and its identification. But the essential nature of C. *diphtheriae* serotypes is not yet well enough known. Eagleton and Baxter (1924) suggested that the types of C. *diphtheriae* were not fixed, and that the structure of the strains concerning the partial antigenic elements differed only quantitatively, not qualitatively. Our own investigations rather negate this opinion, but they are not yet completed, since we have neither exhausted the multitude of types, nor have we made a complete analysis of the antigenic structure of the observed types. A routine typing by agglutination may lead to considering variants such as we obtained, as new types. Similarly, the reversion to phage sensitivity, connected with reversion of agglutinability, may resemble a new type appearance. This may be the reason of certain observed fluctuations in type frequency during an epidemic. We can *a priori* expect that the rise of resistance as well as the reversion to sensitivity may give origin to transitional strains which may be weakly agglutinable, inagglutinable, or re-agglutinable. The analogy with the above mentioned occurrence observed by us during an epidemic is obvious.

The question arises whether or not the observed serologic changes have an epidemiologic importance. There is evidence of the importance of antibacterial immunity, besides the anti-toxic immunity in diphtheria. In this connection, we investigated the phagocytosis of the nonagglutinable variants in the presence of the serum against the original strain. The results are quite distinct: the phagocytosis of the variant R40 in the presence of the anti-*1174* is much weaker than the phagocytosis of the original strain *1174* from which the variant was derived (Table 1). Parallel with the loss in agglutinability, the susceptibility to the action of opsonins vanishes. Thus we may conclude that from an epidemiological point of view, such changes in agglutinability are significant because they can abolish an acquired immunity. Although it may not lead to a second severe disease because of

existing antitoxic immunity, a latent infection or a subclinical process could result, both phenomena of great epidemiological importance.

Summary

Applying homotypic as well as heterotypic adapted phages we obtained resistant strains of C. *diphtheriae* with changed serologic character. The mechanism of these changes, their importance for the rise of new serotypes, and their epidemiological significance are discussed.

TABLE 2

Phagocytosis of Strain 1174 and R40 in the Presence of Serum Anti-1174 (dil. 1:20)

Strain	Serum	Percentage of Phagocytic Cells	Phagocytic Index
1174	anti-*1174*	92	17,6
	normal	56	5,0
R 40	anti-*1174*	80	7,4
	normal	72	4,5

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OBSERVATIONS ON THE SO-CALLED ACTH-RELEASING ACTIVITY OF NEUROHYPOPHYSIAL EXTRACTS IN SAFFRAN AND SCHALLY'S PITUITARY INCUBATION SYSTEM[a]

CLAUDE FORTIER[b]

Saffran and Schally's technique consists in comparing the amount of ACTH liberated into the medium by rat pituitary glands, incubated with or without the principle to be assayed for ACTH-releasing activity (8); the ACTH assay being subsequently carried out by means of another *in vitro* procedure, based on the rate of corticosteroidogenesis by incubated rat adrenal glands (7). The implicit assumption behind this experimental approach is that a significant increase of ACTH in the medium corresponds to a higher level of secretory activity by the incubated pituitary glands, induced by a presumably *physiological* activator.

On the basis of the so-called *ACTH-releasing activity* evidenced by posterior pituitary extracts, and purified fractions thereof, in their *in vitro* system, Saffran and his co-workers concluded that a principle of neurohypophysial origin, possibly related to the *neurosecretory* material found in the hypothalamico-hypophysial tract, is the mediator of the pituitary-adrenocortical activation induced by stress (8, 9). The validity of this conclusion is challenged by the present report on the lack of correlation between this *in vitro* activity of neurohypophysial extracts, on the one hand, the Gomori-positive neurosecretory content of the neurohypophysis and the state of adrenal cortical activity of the donors, on the other.

Methods

Treatment with hypertonic saline was selected as a means of concurrently stimulating the pituitary-adrenocortical axis and depleting the Gomori-positive neurosecretory content of the neurohypophysis, with a view to comparing the *in vitro* activity of neurohypophysial extracts from treated and untreated donors, in Saffran and Schally's pituitary incubation system.

Male Sprague-Dawley rats from the Holtzman Farm, weighing 220–240 gm. were used. Following acclimatization for two weeks to our laboratory conditions (constant lighting and temperature (25 ± 1° C.); Purina Fox-Chow and water ad-libitum), they were distributed into two groups of 9 and 18

animals, to be respectively used as controls and treated with hypertonic saline. The latter procedure consisted in daily intraperitoneal injections (5 ml./100 gm. body weight) of a 5 per cent NaCl solution, which was also substituted for drinking water, for a period of 4 days (5). The 9 surviving animals were killed by decapitation on the 5th day, 24 hours after the last injection. The posterior pituitaries were immediately removed from the skull. Of these, 3 were fixed in Bouin for subsequent paraffin embedding, sectioning at 6–8 μ, and staining with Gomori's *chrome-alum-hematoxylin-phloxin* method (4). The remaining 6 were ground with sand in 0.1 ml. of Krebs-Ringer-Bicarbonate-Glucose-Ascorbic Acid medium for immediate assay in Saffran and Schally's system. The posterior pituitaries from the intact rats were similarly processed, and assayed on the same day. In addition, adrenals and thymuses of experimental and control rats were collected, freed of adhering tissue, and weighed on an analytical balance.

Assays. In accordance with Saffran and Schally's procedure (8), the adenohypophyses from 6 donor rats, killed by decapitation, were quickly removed from the skull, separated from the neural lobe, wiped clean of blood and cut in half. The two halves of each gland were respectively allotted to beakers S and U, containing 1 ml. of Krebs-Ringer-Bicarbonate-Glucose-Ascorbic Acid medium, freshly gassed with 95 per cent 0^2; 5 per cent CO^2. Immediately before incubation, 0.06 ml. of the aqueous posterior pituitary extract to be assayed was added to beaker U. (This aliquot corresponds, in terms of weight/volume, to the amount of pituitary extract originally assayed by Saffran and Schally (8)). Following incubation for one hour in a Dubnoff Metabolic Shaking Incubator, at 38° C., and in a water-saturated atmosphere of 95 per cent 0^2; 5 per cent CO^2, the incubating media from beakers S and U were aspirated off with a tuberculin syringe, and transferred to small tubes (S and U), containing 0.03 ml. of 1N HCl. The pituitaries were weighed to the nearest 0.005 mg. on an analytical balance, and the pituitary incubating media were neutralized with 0.03 ml. of 1N CO^3HNa. Aliquots of 0.150 (U^1) and 0.450 (U^2) ml. ml./100 mg. of adrenal tissue, of the stimulated pituitary medium (U), were run against similar aliquots (S^1 and S^2) of the non-stimulated pituitary medium (S) in Saffran and Schally's 4-point-2 dose *in vitro* assay for ACTH (7). The results, computed and tested for validity by means of Bliss' methods for factorial analysis and analysis of variance (2), were expressed in terms of the relative potency of stimulated to non-stimulated pituitary incubating media[e], with confidence limits (antilogs of $C^2M \pm CtSm$) for odds of 19 in 20.

As a preliminary to the above, the following experiments were carried out in order to verify some characteristics of Saffran and Schally's *in vitro* system: *Assay of ACTH against itself.* In 5 experiments, two doses (10 (U^1) and 30 (U^2) mu./100 mg. of adrenal tissue, in 0.150 and 0.450 ml. respectively, of Krebs-Ringer-Bicarbonate-Glucose medium) of U.S.P. Reference Standard Corticotropin were run against two identical doses (S^1 and S^2) of the same ACTH in the adrenal incubation system.

Release of ACTH from the "non-stimulated" incubated anterior pituitary. In 9 experiments, aliquots of 0.150 (U^1) and 0.450 (U^2) ml./100 mg. of adrenal tissue, of a non-stimulated pituitary medium, were run against 10

(S^1) and 30 (S^2) mu./100 mg. of adrenal tissue, of U.S.P. Reference Standard Corticotropin in the same system.

Comparison of the ACTH-releasing activity of two sets of half-pituitaries. In one experiment, corresponding aliquots of the incubating media of two sets of non-stimulated half-pituitaries were run against each other in the above system.

Effect of incubation on the activity of ACTH. In one experiment, aliquots of U.S.P. Reference Standard Corticotropin, dissolved in Krebs-Ringer-Bicarbonate-Glucose medium and incubated for one hour under the conditions previously described, were run against similar aliquots of a fresh solution of ACTH in the usual fashion.

Comparison of Sayers' and Saffran's assays. 15 anterior pituitaries were removed from the skull, cut in half, and placed by sets of 6 halves in five separate 20 ml. beakers containing 1 ml. of Krebs-Ringer-Bicarbonate-Glucose medium. Following incubation for one hour, the incubating media were aspirated off and pooled. Of the total volume of 5 ml., 1 ml. was set apart for Saffran and Schally's assay, and the remaining 4 were used in Sayers' assay; both assays being carried out simultaneously. For Sayers' assay, the pituitary incubating medium was made up to a volume of 20 ml. with 0.01 N HCl (U^2), 5 ml. of which were further adjusted to 15 ml. (U^1). Aliquots (0.5 ml./100 gm. body weight, I. V.) of U^1 and U^2 were run against 0.5 (S^1) and 1.5 (S^2) mu. of U.S.P. Reference Standard Corticotropin in the classical adrenal ascorbic acid depletion test in 24-hour hypophysectomized rats (10).

Results

Characteristics of Saffran and Schally's system. a—The mean potency ratio of 1.01 ± 0.05 recorded from the assays of ACTH against itself (Table 1) is an indication of the reliability of the adrenal incubating phase of the system.

TABLE 1

Assays of U.S.P. Reference Standard Corticotropin Against Itself

Expected Potency	Observed Potency (Antilog M)	95 Per Cent Confidence Limits	Index of Precision (λ)
1.00	1.08	0.39–3.47	0.174
1.00	1.07	0.29–5.10	0.193
1.00	1.04	0.48–2.36	0.139
1.00	0.81	0.28–1.72	0.151
1.00	1.06	0.58–2.01	0.117

b—The ratio of 1.00, observed in table 2, attests to the comparability of the ACTH-releasing activity of two sets of incubated half-pituitaries, and to the applicability of our correction factor for differences in pituitary weights in S and U.

TABLE 2

Comparison of the ACTH-Releasing Activity of Two Sets of Incubated Half Anterior Pituitaries

Expected Potency	Observed Potency (Antilog M)	95 Per Cent Confidence Limits	Index of Precision (λ)
1.00	1.00	0.79–1.26	0.049

c—As shown in table 3, the amount of ACTH released by incubated anterior pituitaries is relatively constant, and corresponds to 3.17 $\pm$ 0.48 mu. of ACTH per mg.-hour, a value somewhat inferior to that reported by Saffran and Schally (5.36 $\pm$ 0.31 mu. per mg.-hour (8)). This apparent discrepancy may be related to previously observed differences in pituitary ACTH concentration between samples from different populations (Fortier, in press).

TABLE 3

Amount of ACTH Released by the Incubated Anterior Pituitary

ACTH Released mu./mg./hr.	95 Per Cent Confidence Limits	Index of Precision (λ)
2.39	1.04–4.67	0.134
2.18	1.78–2.61	0.039
2.86	1.79–3.98	0.070
2.18	0.77–3.61	0.107
2.06	1.17–2.95	0.072
3.01	1.47–4.80	0.105
3.54	1.89–5.71	0.107
3.57	0.94–9.08	0.172
6.74	4.82–9.80	0.073

TABLE 4

Effect of Incubation for One Hour on the Activity of U.S.P. Reference Standard Corticotropin

Potency (Antilog M)	95 Per Cent Confidence Limits	Index of Precision (λ)
0.56	0.32–0.83	0.086

d—A significant loss of activity (Table IV) resulted from incubation of purified ACTH for one hour.

e—A higher potency ratio (Table V) was recorded for the same pituitary incubate with Saffran's than with Sayers' assay.

In spite of a slight overlap of the 95 per cent confidence limits of the assays, this finding lends support to the hypothesis of Birmingham *et al.* (1) that the steroidogenic/ascorbic acid depleting (S/AA) activity ratio might be higher in *native* than in *purified* ACTH.

TABLE 5

Comparative Assays of an Identical Pituitary Incubate by the Methods of Saffran and Schally and of Sayers, Sayers and Woodbury

Type of Assay	ACTH (mu./mg./hr.)	95 Per Cent Confidence Limits	Index of Precision (λ)
Saffran and Schally..	2.64	1.32–4.61	0.114
Sayers, Sayers and Woodbury	1.01	0.59–1.68	0.186

Effects of treatment of the donors with hypertonic saline. Treatment with hypertonic saline resulted in a marked adrenal cortical activation, evidenced by a 29 per cent increase of the adrenal weight, and a 69 per cent loss of the thymus weight (Table 6).

TABLE 6

Effect of Treatment with Hypertonic Saline on Survival, Body Weight, Adrenal and Thymus Weights, and Gomori-Positive Content of the Neurohypophysis

Treatment	None	Hypertonic Saline
Survival	100%	50%
Initial B.W. (gm.).....	231.9± 9.2	238.6±5.4
Final B.W. (gm.)	240.4± 9.0	185.5±5.6
Adrenals (mg.)	59.6± 1.7	77.0±2.4
Thymus (mg.)	328.1±26.7	100.8±7.9
Gomori-Positive Content ..	+++	Traces

As previously observed by Ortmann (6) and Hild (5), it also resulted in a nearly complete depletion of the Gomori-positive, *neurosecretory* content of the neurohypophysis (Table 6; Plates I and II). When, however, the *in vitro* activity of aqueous posterior pituitary extracts from treated and untreated donors was compared in Saffran and Schally's system, no significant difference was found in the level of this activity, which was equally high in both cases (Table 7).

TABLE 7

Effect of Prior Treatment of the Donor Rats with Hypertonic Saline on the In-Vitro Activity of an Aqueous Extract of the Neurohypophysis in Saffran and Schally's Pituitary Incubation System

Treatment of Donors	Potency (Antilog M)	95 Per Cent Confidence Limits	Index of Precision (λ)
None	16.11	7.62– 82.79	0.089
Hypertonic Saline	20.89	9.02–367.30	0.118

Discussion

Saffran and Schally's procedure for assaying ACTH (7) has been extensively used in this laboratory over the last two years. It has proved both convenient and reliable. Being based on the

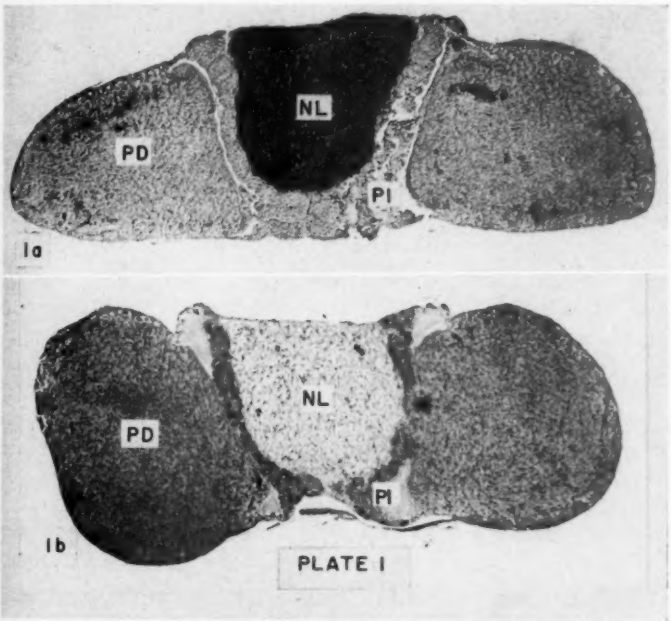

PLATE I. Low magnification (17x) photomicrographs of *chrome-alum-hematoxylin-phloxin* stained transverse sections of the hypophysis of an intact rat (Fig. 1a) and of a rat injected for 4 days with hypertonic saline (Fig. 1b). PD, pars distalis; PI, pars intermedia; NL, neural lobe. Note nearly complete disappearance, in the treated animal, of the darkly stained Gomori-positive material, present in large amounts in the neural lobe of its control.

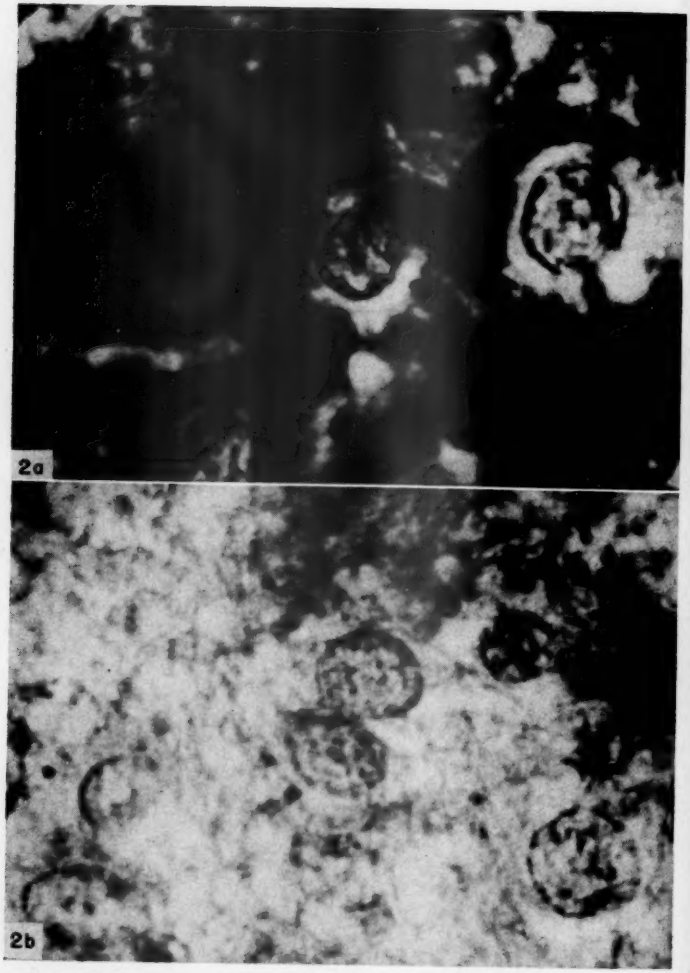

PLATE II. Same preparations at a higher magnification (1600x) to show aspect of Gomori-positive "neurosecretory" material in the neural lobe of the control (Fig. 2a) and its near total absence in the hypertonic saline-injected animal (Fig. 2b).

actual rate of corticosteroidogenesis, it provides a more direct indication of adrenal cortical activity than Sayers' adrenal ascorbic acid depletion test (10). It is highly specific for ACTH; of numerous substances tested, none, but ACTH or pituitary extracts, has been found to increase the rate of corticosteroidogenesis (Fortier and Ward, in press). As an additional advantage, it requires but 20 per cent of the time necessary to carry out an assay by Sayers' procedure. It is more accurate than the latter and offers a wider useful range (rectilinear portion of the log-dose response slope; this range extends from 3 to 300 mu./100 mg. of adrenal tissue in Saffran and Schally's assay, as compared to 0.15 to 2.5 mu./100 gm. B.W. in Sayers'). It is, however, less sensitive (approximately 15 per cent of the sensitivity of Sayers' assay) and, for that reason, not applicable to the determination of ACTH in body fluids. It requires, moreover, rigid timing and strict adherence to the established procedure; the constancy of the pH of the medium, rate of gas flow and degree of atmospheric humidity in the incubating chamber (a Dubnoff Metabolic Shaking Incubator has proved more suitable than the original Warburg apparatus) being especially important with respect to the slope of the assay. Our results, based on the analysis of the last 175 assays carried out in this laboratory, are the following:

Per cent error [100 (antilog Sm-1)]	22.6	±0.7%
S (variance)	12.4	±0.35
b (slope)	114.4	±2.12
λ (index of precision)	0.108	±0.003

It is felt, by contrast, that results based on the utilization of the pituitary-incubation system should be interpreted with extreme caution. As distinct from the adrenal cortex, which does not store its secretory products to any appreciable extent (the small amount of steroids present in the gland is reportedly flushed out during the one-hour preincubation period which precedes the addition to the system of the substance to be assayed (3)), the adenohypophysis is a storage gland. It follows that, whereas the rate of steroid release from the incubated adrenal truly corresponds to its secretory activity, the liberation of ACTH from the pituitary may simply reflect passive leakage of the hormone. Moreover, whereas steroids are stable compounds, ACTH is readily inactivated by incubation (Table 6).

Five possibilities may theoretically account for the detection of a significant increase of ACTH in a pituitary incubating medium to which a given principle has been added. It may result from:

1—presence of ACTH in the added principle;

2—potentiation of the response of the incubated adrenals to the ACTH released by the pituitaries (*native* ACTH);

3—inhibition of ACTH inactivation in both pituitary and adrenal incubating systems;

4—enhanced release (whether active or passive) from the incubated pituitaries;

5—a combination of the above.

The present study was not aimed at defining each of these modalities with respect to the neurohypophysial extract considered. Our object was to determine whether the *in vitro* activity of this extract, in Saffran and Schally's system, is related to 1) the amount of *neurosecretory* material present in the neural lobe, 2) the state of adrenal cortical activity of the donors. An experimental situation was therefore selected which resulted in near-total depletion of the *neurosecretory* content of the neural lobe, on the one hand, and marked adrenal cortical activation, on the other. Our failure to ascertain the postulated relationship raises additional doubts as to the physiological significance of findings based on the utilization of Saffran and Schally's pituitary incubation system.

Two essential prerequisites would be needed to substantiate the claim that a principle, active in this system, is the mediator of the pituitary-adrenocortical activation induced by stress:

1—The elucidation of its mode of action (whether it involves release of ACTH, contamination with ACTH, potentiation of its effects, or inhibition of its inactivation);

2—The demonstration that the increased release, if ascertained, corresponds to enhanced secretory activity of the pituitary, as opposed to passive leakage of the hormone through its cells. This could be indirectly achieved by showing that the principle considered activates the pituitary-adrenocortical axis in an otherwise unresponsive in-vivo preparation, such as the hypothalamic-lesioned or hydrocortisone-inhibited animal.

Summary

In order to verify Saffran and Schally's suggestion, based on the utilization of an *in vitro* pituitary incubation system, that a principle of neurohypophysial origin, possibly related to the *neurosecretory* material found in the hypothalamico-hypophysial tract), is the mediator of the stress-induced activation of the pituitary-adrenocortical axis, rats were daily injected with hypertonic saline for a period of 4 days. At the end of this time adrenal and thymus weights, Gomori-positive content of the neurohypophysis and so-called *ACTH-releasing activity* (enhancement of the adrenocorticotrophic activity of the pituitary incubation medium) of an aqueous extract of this gland, in Saffran and Schally's *in vitro* system, were determined. Treatment with hypertonic saline resulted in near-total depletion of the *neurosecretory* content of the neural lobe, on one hand, marked adrenal cortical activation, on the other. The *in vitro* activity of the neurohypophysial extracts bore no relationship to the treatment of the donors, their state of adrenal cortical activation or the *neurosecretory* content of the neural lobes. In the light of these results and of theoretical considerations, the physiological significance of findings based on the utilization of Saffran and Schally's pituitary incubation system is seriously questioned.

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[b] Neuroendocrine Unit, Blue Bird Neurological Research Laboratories, Methodist Hospital, and Department of Physiology, Baylor University College of Medicine, Texas Medical Center, Houston, Texas. The author is indebted to Dr. and Mrs. Walther Hild of the Tissue Culture Laboratory of The University of Texas, Medical Branch, Galveston, for their helpful advice in connection with Gomori's *chrome-alum-hematoxylin-phloxin* staining method, and to Dr. Murray Saffran of the Allan Memorial Institute of Psychiatry, Montreal, Canada, for guidance in the utilization of his *in vitro* techniques. The able technical assistance of Mrs. Carol Smith, Miss Ruth C. Wood and Mr. Paul Bruchstein is gratefully acknowledged.

[c] The slight difference in the pituitary weights of S and U was taken into account, and assuming a linear relationship between pituitary weight and response over the range of this weight discrepancy, the values recorded for U^1 and U^2 were multiplied by the following Correction Factor:

$$CF = \frac{a + bx}{a + [bx(\log \bar{U}/\bar{S})]}$$

Where a = position of the dosage-response curve at $\bar{x}$, equal numerically to $\bar{y}$ for all dosage levels included in the curve; b = slope of the straight dosage response line; x = log-dose against which the response can be plotted as a straight line; S = weight of the non-stimulated pituitaries; U = weight of the stimulated pituitaries.

AN EVALUATION OF THE ROLE OF MOLDS IN THE COMPARATIVE BIOCHEMISTRY OF CARBOHYDRATE OXIDATION[a]

J. W. FOSTER

Even the most dedicated student of mold metabolism will concede that the discovery and elucidation of mechanisms of carbohydrate utilization and cycles of respiration have been made in yeast, bacterial or mammalian cells. Fungi have been conspicuous by their absence. Once discovered, these mechanisms fairly quickly were shown to be operative throughout biological systems, thereby becoming part of our fundamental knowledge of living systems. It was largely from respiratory mechanisms that the Kluyverian concepts of comparative biochemistry evolved. This concept tells us that regardless of the biological system in which the discovery is made, the probability is great that new mechanisms will prove to be of general significance in biology.

As late as twenty years ago, the remarkable successes in elucidation of the Embden-Meyerhof-Parnas mechanism of glycolysis engendered a complacency that here was THE pathway of hexose breakdown. And, parenthetically, if because of its display of alcoholic formation yeast was deemed suited for studies on the mechanism of glycolysis, it may not be amiss in this connection to remind ourselves that Pasteur himself described the occurrence of a true alcoholic fermentation in perfectly respectable filamentous fungi. Conceivably, our knowledge of glycolysis might just as well have come from such a fungus.

In the past five years conclusive evidence has been obtained for the existence of anaerobic pathways of carbohydrate breakdown distinctly different from the Embden-Meyerhof-Parnas scheme (17, 21, 26, 28, 39, 61, 62, 63), and it does not seem too risky to adopt the view that additional anaerobic pathways are yet to be revealed. Although aerobic mechanisms might seem to be a separate and distinct category from anaerobic mechanisms, they are not fundamentally so, in the sense that the difference is oxygen versus an organic metabolite as the ultimate hydrogen acceptor. Thus, the anaerobic oxidation of glucose by the bacte-

rium *Pseudomonas lindneri* (26) appears to be the same as that occurring aerobically in *Pseudomonas saccharophila* (21). Similarly, in numerous systems the aerobic oxidation of carbohydrate via the 6-phosphogluconate pathway has a counterpart in the oxidation of glucose anaerobically by *Leuconostoc mesenteroides* (28). This point is instructive, for it means that the implications of aerobic pathways of carbohydrate metabolism are by no means restricted to aerobic organisms or processes.

The age of aerobic metabolism of carbohydrates began with the tricarboxylic acid cycle (TCA) proposal of Krebs and Johnson in 1937. Vital parts of this cycle are the 6-carbon tricarboxylic and 4-carbon dicarboxylic acids. Now, in virtually all aerobic systems possessing the TCA cycle, these acids normally do not accumulate nor are they present in more than trace amounts. As intermediates in carbohydrate oxidation they are transformed as fast as they are formed. The brilliant concept of the cycle stemmed from the catalytic effects of the various cycle components on oxidation of pyruvate and carbohydrate, on their rates of utilization, on their interconvertibility, and upon demonstration of each of the individual enzymes of the cycle in pigeon liver and breast muscle (Krebs, 1954).

Discovery of the participation of the di- and tricarboxylic acids was, therefore, the outcome of investigations of the mechanism of aerobic oxidation of carbohydrate. Is it straining credulity to hold that the converse could well apply, namely, that an investigation of the di- and tricarboxylic acids would have led to the discovery of the Krebs cycle of carbohydrate oxidation? I think not, and my chief purpose is to develop this theme, especially in relation to fungi. One might suggest that there was little reason to suspect the di- and tricarboxylic acids of having a unique role in carbohydrate metabolism. But there was a reason, a very cogent reason, for suspecting this in the case of certain filamentous fungi that had long been known to produce from carbohydrates extraordinary accumulations of citric acid and of fumaric, succinic and malic acids. Actually, the distinctive accumulation of citric acid in cultures of Penicillia was known for more than 40 years before the postulation of the Krebs cycle. Moreover, it was early recognized that this was a consequence of carbohydrate oxidation, since carbohydrate was commonly the substrate in these experiments. Furthermore, oxygen was known

to be indispensable, the citric acid once formed was subsequently transformed, and citric acid was interconvertible with mixtures of other carboxylic acids now known to be components of the TCA cycle. Who can deny that those distinctive organic acid accumulations might have been beacons pointing the way to carbohydrate oxidation pathways? Indeed, Raistrick and Clark in 1919 proposed as the key mechanism in citrate synthesis the now accepted acetate plus oxalacetate condensation. As for the other reactions of the cycle, the enzymes for virtually every one have been found in *Aspergillus niger* and the extensive isotope data are consistent with their operation, too (Chain, 1956).

Corresponding situations existed in connection with the distinctive accumulations of fumaric acid from carbohydrate by *Mucor stolonifer (Rhizopus nigricans)* first described by F. Ehrlich in 1911. Here, too, one can hardly fail to be intrigued by what might have evolved in the way of carbohydrate oxidation cycles had the biochemistry of this unique system been investigated intimately.

We have already alluded to the import of the direct oxidation of glucose via the hexosemonophosphate pathway as a mode of glucose utilization alternative to glycolysis. In retrospect, how do fungi stand as beacons of this alternate metabolic chain? Suffice it to remark that gluconic acid accumulation in distinctive amounts in mold cultures has spotlighted this potential route of carbohydrate oxidation ever since Molliard discovered this phenomenon fourteen years ahead of the biochemical proof by Warburg, Lipmann and Dickens. The operation of the complete cycle in other fungi is a fairly well established fact (16, 32).

I have cited golden, albeit lost, opportunities the mold has had to contribute profoundly to basic pathways of metabolism of carbohydrates. They embody, I think, a rationale which ought to provide valuable insight and guidance in the discovery of new biochemical mechanisms of wide biological significance. The gist of this rationale is that the citric and gluconic acid pathways operate in the great majority of animal, yeast, plant, bacterial and mold cells, but normally without distinctive accumulations of any of the components of the respective cycles. We now understand that in fungi where distinctive product accumulation occurs, it denotes simply an interrupted, or more likely, an imbalanced cycle. It seems an entirely reasonable assumption that

the reverse would hold true, namely, that the distinctive accumulation of products of carbohydrate metabolism in fungus cultures signifies an interruption of a corresponding oxidation pathway. Whereas the tell-tale evidence of existence of new metabolic pathways in the great majority of other organisms cannot be foretold without laborious efforts and tenuous deductions from isotope studies, from poisoning experiments, from cell-free enzymatic studies, from mutants, and from inconsistencies of new data with older theories, it seems to be uniquely characteristic of fungi that they virtually advertise the presence of new metabolic pathways by the piling up of compounds representing one or more stages in a pathway.

Fungi seem to be particularly liable to the imbalances which result in these pileups, through operation of what has been called shunt metabolism (Foster, 1949). Many strains of different fungi may accumulate one or more carbohydrate breakdown products in an appropriate nutritional environment, but seemingly common to most of these instances is the presence of a relatively high concentration of substrate, at a time when cell synthesis cannot, or is not allowed to, proceed at the maximal rate. The balance between assimilatory mechanisms and oxidation mechanisms resulting in accumulation of oxidation products is especially liable to disturbance, so that one can be greatly favored at the expense of the other. Just why this trait is particularly striking in fungi is a mystery, but it may have to do with the subcellular architecture of the fungal cell and the location of particular families of enzymes. Also, this inverse relation becomes easier to interpret in terms of recent evidence from various microorganisms (36, 43, 47) that the TCA cycle is primarily a mechanism for furnishing intermediates for biosynthesis and assimilation purposes.

Mycological production of ethanol, of citric acid, of fumaric acid and of gluconic acid were ahead of their times, from the standpoint that methods, concepts, and contextual experiments were not yet favorable for their exploitation. More important at the present time is whether we can capitalize on these experiences to recognize new pathways signaled by beacons analogous to those unheeded in the past. The merit of the fungi in exposing profitable new areas of carbohydrate metabolism is already on the verge of repeating history in at least a few other instances.

The production of high yields of itaconic acid from carbohy-

drate by certain species of Aspergilli has been known for almost thirty years (31). Except for attractive prospects as a possible polymerization raw material for commercial plastics, this peculiar fermentation was virtually ignored for 24 years. But, quite in agreement with the premise developed above, as soon as the itaconic mechanism was examined by Tatum and Corzo in 1953 (15), results of unusual interest developed. It is now clear (6–9) that Kinoshita's (31) assumption is correct that itaconate arises via decarboxylation of *cis*-aconitic acid derived from citric acid synthesized via the TCA cycle (Fig. I). However, isotopic ex-

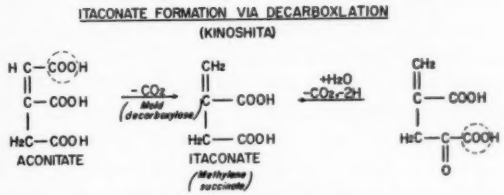

FIG. I. Left portion, Kinoshita's mechanism. Right portion, Korkes' mechanism of oxidative decarboxylation of γ-methylene, α-ketoglutaric acid.

periments with intact cells indicated that the formation of itaconic acid from citrate seemingly was inconsistent with the known tricarboxylic acid cycle reactions. Unexpected was the finding that the carboxyl group lost by enzymatic decarboxylation of *cis*-aconitic acid was not the one predicted from the Krebs cycle. The prediction assumed that it was the carboxyl adjacent to the double bond that was lost. This is represented as carbon 1 in figure II. However, the mechanism has been clarified very recently by Bentley and Thiessen (9) in a superb study (7, 8) of the intermediary metabolism of *Aspergillus terreus*. By ingenious use of radioactive carbon and deuterium they showed that the assumption was erroneous that aconitic decarboxylase eliminates the carbon adjacent to the double bond in *cis*-aconitic acid. They proved that the carboxyl group lost was that represented as carbon-5 in figure II. It is the carboxyl of the original acetate. During removal of carbon-5 by the decarboxylase, an electron shift occurs in the carbon skeleton, resulting in a concomitant shift of the double bond from the 2–3 position to the 3–4 position. The product is itaconic acid.

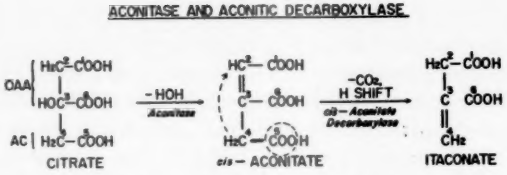

FIG. II. Left portion, formation of *cis*-aconitate by dehydration of citrate. Right portion, formation of itaconate from *cis*-aconitate by decarboxylation and hydrogen shift (Bentley and Thiesen, 9).

Without doubt, the accumulation of large yields of itaconic acid by these fungi signifies an unbalancing of a pathway of oxidation in which itaconate is an intermediate. Clarification of the mechanism of dissimilation of itaconic acid is all that stands in the way of bestowing recognition on a new cycle of biological oxidation, an "itaconate cycle."

Respiratory cycles play a major role in furnishing cells with the precursors of cellular components such as amino acids, lipids, nucleic acids, etc. Even before the missing stages of the "itaconate cycle" are filled in, it seems reasonable to assume that this cycle likewise will play a role in furnishing precursors of one kind or other. It may not be premature to note that glutamic acid synthesized by the anaerobic bacterium *Clostridium kluyveri* cannot, according to the experiments of Tomlinson (58), arise via ∝-ketoglutaric acid formed by the Krebs cycle. And in a related bacterium, *Clostridium tetanomorphum*, Barker and co-workers (5) have shown that the straight carbon chain of glumatic acid is both synthesized and degraded via mesaconic and citramalic acids. These intermediates are branched chain 5-carbon dicarboxylic acids very closely related to itaconic acid, as figure III shows. Of special significance are the enzymological data revealing a new route to biosynthesis of these compounds. A condensation takes place between 1 molecule of acetate and 1 of pyru-

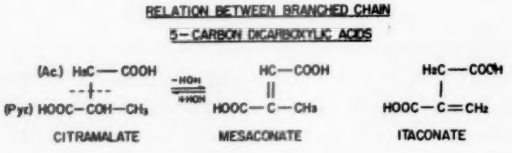

FIGURE III

vate in a manner entirely analogous to the condensation between acetate and oxalacetate in synthesis of citrate.

In all probability, the Krebs and the itaconate pathways of oxidation are not mutually exclusive in any one organism. They may operate simultaneously, or one may be utilized preferentially according to the conditions. For example, Eimhjellen and Larsen (20 have shown that when growth of *Aspergillus terreus* takes place in a medium near neutrality, the conventional Krebs cycle enzymes are formed, the organic acids accumulating being different than when the fungus is cultivated in acidic medium. In the latter, the itaconic mechanism predominates. Perhaps the relative proportionation between the two pathways may ultimately have as a basis the recent discovery by Nielson (44, 45) that in *Aspergillus niger* aconitase, heretofore believed to be a single enzyme, is two separable and distinct enzymes. One, similar to animal aconitase, produces both citrate and isocitrate from *cis*-aconitate. The other, called "aconitic hydrase," forms exclusively citrate from *cis*-aconitate.

Before departing from the intriguing new potentials of itaconate metabolism, the suggestions of Korkes (33) are worth noting. Growing recognition of the distribution of γ-methylene glumatic acid and its deamination product, γ-methylene ∝-ketoglutaric acid, especially in plants (27), led Korkes to point out that an oxidative decarboxylation of the latter compound, analogous to that occurring with ∝-ketoglutaric acid in the Krebs cycle, would yield methylene succinic acid, that is, itaconic acid, instead of succinic acid (Fig. I). The validity of this hypothetical mechanism of itaconate biosynthesis remains to be established experimentally.

A subject nowadays unfolding swiftly as a biochemical mechanism of generalized significance is the utilization of 2-carbon compounds, namely acetate or the acetyl equivalent, as the sole source of carbon in growth and respiration. This situation poses a special problem for the cell even though the substrate acetate here is equivalent to that formed via pyruvate when glucose is the substrate. In either case, condensation with oxalacetate may occur yielding citrate, and potentially the conventional Krebs cycle could occur. However, in spite of their similarity with respect to acetate oxidation, a vital difference exists between these situations. The crux of this lies in the regeneration of the oxalace-

tate as the acceptor for acetate to form citrate. When oxidizable acetate arises from pyruvate, acceptor oxalacetate is constantly produced by a carbon dioxide fixation reaction involving some of the pyruvate. In organisms growing on acetate only, a tricarboxylic acid cycle can be considered only if some mechanism for regeneration of acceptor oxalacetate can be found other than β-carboxylation of pyruvate. Obviously, any such new mechanism would have to involve oxalacetate synthesis from 2-carbon molecules. An observed relation between utilization of acetate and appearance of succinic acid in mammalian and yeast cells led Thunberg (56), Knoop and Wieland independently to postulate in the 1920's a direct dehydrogenative condensation of 2 molecules of acetate (Fig. IV).

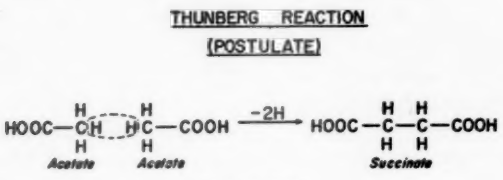

FIGURE IV

Nowhere has the subject of utilization of 2-carbon compounds been more extensively investigated through the years than in the filamentous fungi, especially in those capable of yielding distinctive accumulations of di- and tricarboxylic acids when acting on carbohydrate substrates. The historical aspects of this subject have been collected elsewhere (22), but an enormous amount of investigation has been done through the years dealing with 2-carbon precursors of the C_4 and C_6 carboxylic acids in fungi. This stemmed from the observed relation between the initial formation of 2-carbon substances, especially ethanol and acetate, during carbohydrate metabolism, and their subsequent conversion to the carboxylic acids. Hence, it seemed reasonable to study carboxylic acid formation in the absence of carbohydrates, but with the supposed intermediate 2-carbon compounds instead. As far back as 1904 Mazé and Perrier (42) showed that ethanol could be converted to citric acid in mold cultures, and this approach to the study of mycological formation of acids from carbohydrates was the main one employed through the years in the laboratories of

Chrzaszcz, of Bernhauer and of Butkewitsch, to mention a few of those prominently connected with this field. Almost invariably the Thunberg postulation (56) was invoked to explain the biosynthesis of C_4 and C_6 acids from 2-carbon substrates by fungi, and this interpretation was extended, not illogically, to account for the biosynthesis of these higher acids from carbohydrates. A conspicuous need arose for some very efficient reaction mechanism to account for the biosynthesis of C_4-dicarboxylic acids from acetate. Proof that the methyl carbons of acetate (or ethanol) do indeed become joined to form the methylene carbons of succinate, was conclusively established by means of isotopes in a fumaric acid-yielding strain of *Rhizopus nigricans* (23, 24). Although the precise steps of this novel biochemical condensation were not clarified, the Thunberg reaction was deemed most probable and the data obtained with *Rhizopus nigricans* were not inconsistent with it.

Considering the very widespread importance ascribed to the Thunberg reaction as a mechanism for direct synthesis of C_4 dicarboxylic acids, convincing enzymatic experiments in its support have been wanting. Despite some limited suggestions in its behalf (51, 59), a growing skepticism seems to prevail as to its being the specific mechanism involved. Furthermore, a very recent discovery of a different mechanism of condensation of 2-carbon compounds precludes dependence on the Thunberg reaction, and at once provides a very probable explanation for diverse metabolic phenomena. Although the stage has been long set for providing the answer to this puzzle through the huge amount of work done with fungi, where the problem is revealed in its sharpest focus, the solution was provided largely through studies with bacteria. The huge backlog of information on fungi seemingly fits into this new scheme. In fact, henceforth, any discussion of fungi in relation to oxidation pathways will not be complete without consideration of what has come to be known as the glyoxylate cycle (Kornberg and Krebs, 1957).

Elucidation of this cycle was aided by studies of breakdown of isocitric acid by certain bacterial extracts. Whereas in some systems aconitic acid and isocitric acid may respectively be decarboxylated and dehydrogenated as described in the previous sections, in others, including bacteria, and at least one fungus, the isocitric acid is split enzymatically to glyoxylate and succinate

(13, 46, 48, 49, 54, 55). The names isocitratase and isocitric lyase have been proposed for this cleavage enzyme. The key reaction of the glyoxylate cycle was exposed by the discovery by Wong and Ajl (60) of a new bacterial enzyme called "malate synthetase." With appropriate coenzymes, malate synthetase synthesizes malic acid by condensation of two 2-carbon precursors, namely, acetate and glyoxylate. Kornberg and Krebs (34) have shown that isocitratase and malate synthetase operate concomitantly in bacterial extracts. Oxidation of acetate in these systems resulted in the synthesis of one mole of succinate from 2 moles of starting acetate. This is precisely what the hypothetical Thunberg reaction was supposed to accomplish. However, these investigators have demonstrated elegantly that oxidation of acetate to succinate is not a simple one-step reaction, but involves multiple enzymes functioning in an integrated cyclic fashion. The oxidation of acetate to succinate in the extracts studied did not occur unless catalytic amounts of isocitric acid were present. Steps in the cycle were shown to be the following:

Acetate + oxalacetate	→ citrate
Citrate	→ isocitrate
Isocitrate	→ succinate + glyoxylate
Acetate + glyoxylate	→ malate
Malate + ½ O_2	→ oxalacetate
Sum: 2 Acetate + ½ O_2	→ succinate

This pathway bids well to rival the tricarboxylic acid cycle as a means of supplying intermediates for biosynthesis of amino acids. It may be noted that glycine can be synthesized by transamination of one of the key compounds in the cycle, glyoxylic acid (49, 57). It will be seen from figure V that the glyoxylate cycle is in reality a citric acid cycle with a different device for regenerating the acetate acceptor oxalacetate, namely, by the malate synthetase reaction. Continuous regeneration of oxalacetate requires a continuous source of glyoxylate, and this is provided by the enzymatic cleavage of isocitrate. Thus, when acetate is the sole source of carbon, the vital acceptor oxalacetate is regenerated by glyoxylation of acetate, instead of by carboxylation of pyruvate, as when carbohydrate is the substrate.

Association of glyoxylic (and glycolic) acids with the formation and utilization of acetate and C_4-dicarboxylic acids in fungi

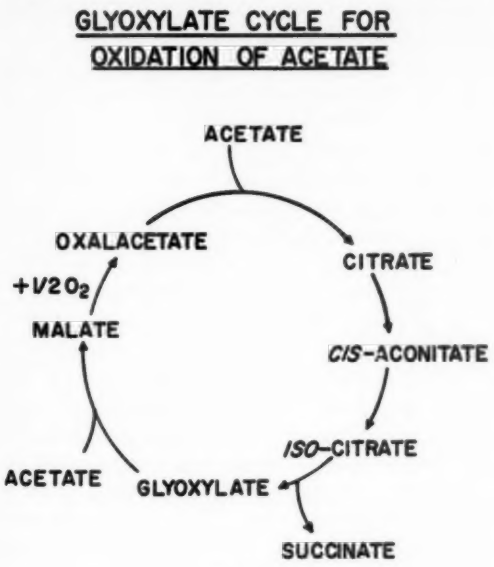

FIGURE V

was well recognized by Bernhauer in 1939 (10, 11). More recently, Olson (46) has shown that extracts of *Penicillium chrysogenum* contain enzymes for both the Krebs and the glyoxylate pathways of isocitrate metabolism, namely, those effecting an oxidative decarboxylation to α-ketoglutarate, and those effecting a cleavage to succinate and glyoxylate. As Kornberg and Krebs (34) have pointed out, virtually all previous studies of oxidation of ethanol and acetate by molds are in agreement with the operation of the glyoxylate cycle. Without doubt, mold metabolism is intimately interwoven in the evolution of our ideas relative to the glyoxylate cycle, even though much remains to be clarified as to its role in fungi. Unless new evidence in behalf of the Thunberg reaction is forthcoming, it soon may well have outlived its usefulness in the explanation of organic acid synthesis in fungi.

There are two other reactions involving C_4-dicarboxylic acids which warrant inclusion here. Both are, for the moment, known to occur only in fungi, but if the previously discussed types of

metabolism are any indication, comparable pathways probably exist in non-fungal systems.

The first is *trans*-L-epoxysuccinic acid, which accumulates as a product of carbohydrate metabolism of certain strains of at least three different mold species (40). This compound is closely related chemically to the dicarboxylic acids of the Krebs cycle. According to the theme of this paper, accumulation of this acid in distinctive amounts would indicate that the acid is formed as an intermediate in a new modification of a respiratory or oxidation cycle. It is too early to tell if it is a version of the citric acid cycle, or of the glyoxylate cycle, or in itself represents a new and distinct mode of biosynthesis of C_4-dicarboxylic acids. We do know that the epoxy acid is synthesized from ethanol or acetate as the sole carbon source. Whether the epoxy acid is formed via malate initially generated by "malate synthetase" or via a new version of a 2 C_2 condensation remains to be seen. A precursor role for malate seems inviting since epoxysuccinate formation would represent a new type of oxidation of malate alternative to the classical one that yields oxalacetate. The difference would lie in whether the dehydrogenation occurs at a single carbon atom or at two (Fig. VI). It may be significant that washed cells of the epoxysuccinate mold formed considerable yields of epoxysuccinate from added malate, but not from succinate or fumarate.

Strong evidence for a role of epoxysuccinate in a new oxidation cycle lies in the fact that the epoxysuccinate-producing strain of *Aspergillus fumigatus* is capable of metabolizing the epoxysuccinate to *meso*-tartaric acid (40). This reaction, a hydrolysis (Fig. VII), can readily be accomplished with living cells or with

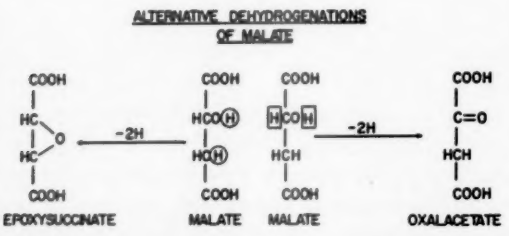

FIG. VI. Left portion, oxidation of malate by loss of 2 hydrogens at 2 different carbon atoms. Right portion, oxidation of malate by loss of 2 hydrogens at a single carbon atom.

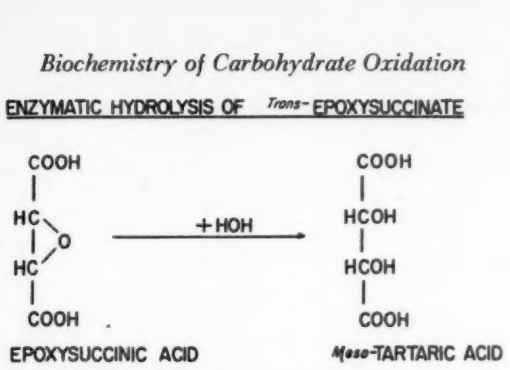

FIG. VII. Hydrolysis by *trans*-L-epoxysuccinic hydrolase.

cell-free preparations of an enzyme named *trans*-epoxysuccinic hydrolase.

This apparently is the first instance of the biological occurrence of *meso*-tartaric acid.

The hydrolase has been found in a great variety of fungi, bacteria and yeasts, isolated from soil. One could logically infer that the epoxysuccinate-*meso*-tartrate system plays a role in the metabolism of the organisms concerned. *Meso*-tartrate ordinarily does not accumulate in the cultures attacking epoxysuccinate, indicating a rapid further utilization of the tartrate. In these and in many other systems, bacterial and animal, it is now well-established that *meso*-tartrate is rapidly oxidized (38, 41, 50, 52). In bacteria, a stereospecific dehydration to oxalacetic acid occurs (52), achieving an entrance to known pathways of oxidation. Alternatively, in animal mitochondria *meso*-tartrate is dehydrogenated to oxaloglycolic acid (37). These reactions are illustrated in figure VIII.

Although many gaps remain to be filled, one can perceive developing from studies with fungi an oxidative pathway of metabolism which would bypass the direct oxidative formation of oxalacetate from malate by means of malic dehydrogenase. The net effect of the epoxysuccinate pathway (Fig. IX) would be the same—oxalacetate is produced from malate. Here would be a novel means of generation of an α-keto acid from the corresponding hydroxy acid. In reality, it provides a means of synthesis of oxalacetate via hydroxyfumaric acid. The latter is the enol tautomer of oxalacetate. Spontaneous chemistry, a tautomeric shift, is therefore the trick the organism employs to remove the last

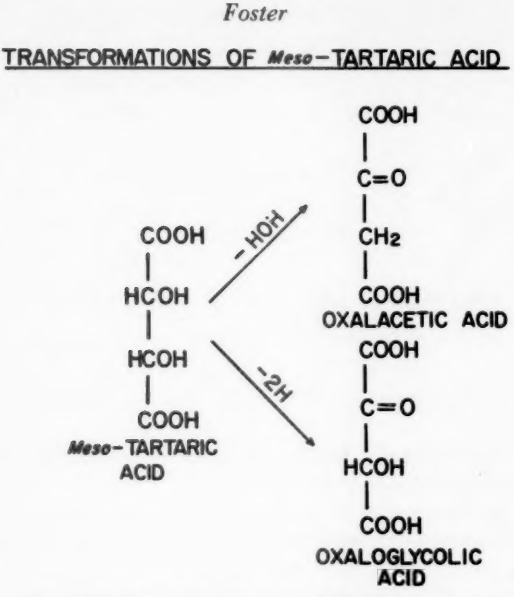

FIG. VIII. Upper portion, tartrate dehydrase action. Lower portion, tartrate dehydrogenase action.

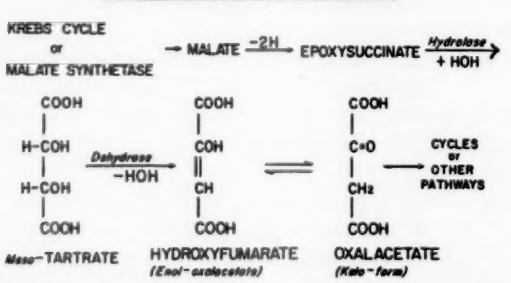

FIG. IX. New pathway of oxalacetate formation from malate via epoxysuccinate, *meso*-tartrate and hydroxyfumarate as precursors.

hydrogen atom from the α-carbon atom. Thus, the traditional malic dehydrogenase of the Krebs cycle produces keto-oxalacetic acid from malate; the epoxysuccinate-tartrate pathway produces enol-oxalacetic acid from malate. In solution, both forms exist in equilibrium mixture.

Considering the widespread occurrence of the stereoisomers of tartaric acid in the plant and microbial kingdoms, and the widespread existence of enzymes capable of attacking the tartrates, the operation of specific pathways involving tartrate in intermediary metabolism becomes more and more likely. Mechanisms of formation of the optically active stereoisomers of tartrate offer additional opportunities for oxidation cycles.

The second of the new reactions involving C_4-dicarboxylic acids has to do with another of the classical organic acid accumulations in fungi and in plants, namely oxalic acid. Before the turn of the century, De Bary and later Wehmer regarded oxalic acid in much the same light we do here, that is, as a product of incomplete carbohydrate oxidation. According to the rationale being followed in this exposition, the accumulation of oxalate would signify a new pathway of intermediate metabolism. Much speculation has attended proposals as to the mechanism of formation of oxalic acid, but it remained for enzymological experiments reported only last year by Hayaishi and co-workers (29), to show how oxalate is formed in *Aspergillus niger*. These findings render inevitable the existence of still another modification of the carboxylic acid cycle. Oxalacetic acid was shown to undergo a hydrolytic cleavage by an enzyme named oxalacetic hydrolase (Fig. X). This mechanism had been suggested by Raistrick and Clark almost 40 years ago (22).

Mold metabolism is, therefore, responsible for the discovery of an extremely important biochemical fact of life. It provides the

FORMATION OF OXALIC ACID

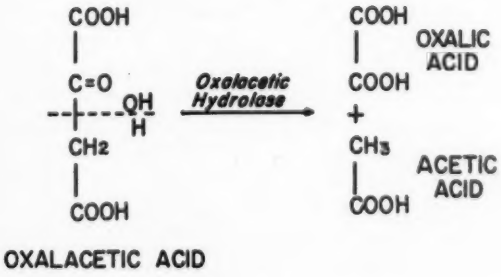

FIGURE X

first instance of a biological cleavage of a C_4-dicarboxylic acid, to two 2-carbon fragments. At once this opens new possibilities for operation of modified oxidation cycles, since oxalate may be further oxidized or decarboxylated to CO_2 (25, 53). Hydrolytic cleavage of oxalacetate very likely ties in with other oxidation cycles in oxalate-producing systems. An oxalate cycle of oxidation would provide a means of disposal of the products of all of the principal cycles already discussed, namely, the Krebs cycle, the glyoxylate cycle and the epoxysuccinate cycle. Succinate is the product of the Krebs cycle that enters the oxalate cycle; succinate and glyoxylate are the products of the glyoxylate cycle; and oxalacetate is the product of the epoxysuccinate cycle. The oxalate cycle outlined here is, in reality, a cyclic mechanism for the oxidation of glyoxylic acid. One molecule of acetic acid is the acceptor for a molecule of glyoxylic acid, which becomes oxidized to oxalic acid while coupled to the acetate. The splitting out of oxalate means a concomitant regeneration of the acceptor acetic acid. The individual reactions are these:

Acetate + glyoxylate	→ malate
Malate + ½ O_2	→ oxalacetate
Oxalacetate + HOH	→ acetate + oxalate
Sum: Glyoxylate + ½ O_2	→ oxalate

Integration of the oxalate cycle with other respiratory cycles involving organic acids is depicted in figure XI.

Detailed analysis of additional mold "fermentations" is not feasible here, but this exposition would be incomplete if it did not

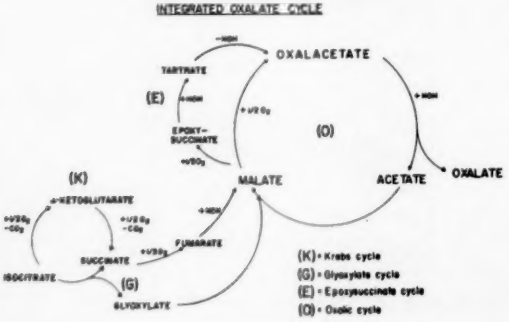

FIGURE XI

consider the formation of kojic acid in large yields by certain fungi. In the light of the proved metabolic implications of the accumulation of "shunt" substances derived directly from carbohydrate oxidation, it is not presumptuous to conclude that clarification of the mechanism of formation and transformation of kojic acid will unveil a new cycle of carbohydrate oxidation. The same may be said for the question of a probable new route to the biosynthesis of cellular components via a kojic acid pathway. An especial intrigue attends this particular process, because the preponderance of isotopic evidence indicates that kojic acid formation represents a unique direct pathway of oxidation of the hexose molecule without rupture of the 6-carbon chain (2, 3, 4, 18, 19). One possibility is that the kojate pathway is an extension of the gluconate pathway discussed earlier. On the other hand, it may represent a completely different alternative. In either case, kojic acid is an oxidation product of glucose in the pyranose form (Fig. XII).

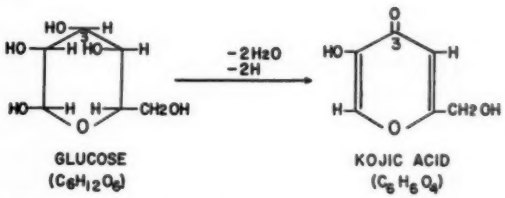

FIG. XII. Relation of kojic acid to glucose. Note dehydrogenation at number 3 carbon atom.

Of particular interest is the theory that one of the first steps in this oxidation would involve a dehydrogenation of the number 3 carbon atom of glucose. This would be novel, for the number 2 carbon or number 5 carbon is oxidized in the other known direct oxidative pathways, yielding 2- and 5-ketogluconic acid respectively. The intriguing question of the existence here of a 3-keto intermediate can be resolved only by experiments.

Finally, it is pertinent here to mention the report by Walker and co-workers (12) of formation of substantial yields of glucosone from glucose by certain *Aspergillus* species (Fig. XIII). This, too, represents a new direct oxidative pathway of glucose, and is considered by Ikeda (30) to be a possible intermediate in the biosynthesis of kojic acid and other γ-pyrones.

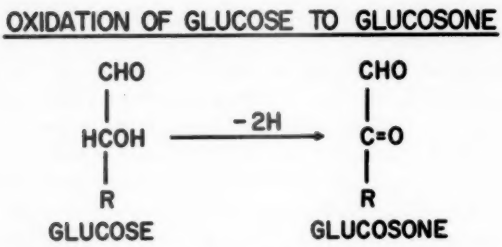

FIG. XIII. Alternative pathway of oxidation of glucose.

Many additional instances of accumulation of mold products could be used to implement the line of reasoning developed throughout this paper. When one thinks of how few fungi have been intensively scrutinized along these lines, new possibilities seem inevitable. In view of the record just covered, such product accumulations almost certainly will signal the operation of new aspects of carbohydrate oxidations.

It is clear, then, that accumulated shunt products in the fungi could well have played an historic role in the elucidation of all the currently known major mechanisms of carbohydrate oxidation. Current events make it equally clear that shunt accumulations in fungi are now assuming a position of recognition in such elucidation. By extrapolation, in the future, shunt accumulations in fungi seem destined to assume leadership in discovery of new aspects of comparative biochemistry. The final assessment of the relative importance and distribution of any pathways discovered in molds in this manner depends, of course, upon investigations on other microorganisms, on animal cells and on plant cells.

The coordination of information obtained in this way is the quintessence of comparative biochemistry. The opportunities available in the fungi, and the situation as it exists in these organisms, are admirably epitomized in the following lines from Aristotle (1):

"The investigation of the truth is in one way hard, in another easy. An indication of this is found in the fact that no one is able to attain the truth adequately, while, on the other hand, we do not collectively fail, but every one says something true about the nature of things, and while individually we contribute little or nothing to the truth, by the union of all a considerable amount

is amassed. Therefore, since the truth seems to be like the proverbial door, which no one can fail to hit, in this respect it must be easy, but the fact that we can have a whole truth and not the particular part we aim at shows the difficulty of it."

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CHANGES IN ENZYME ACTIVITY *(GLUTAMIC OXALOACETIC TRANSAMINASE, LACTIC DEHYDROGENASE, CYTOCHROME C, AND CYTOCHROME OXIDASE)* IN SERUM AND HEART MUSCLE AFTER EXPERIMENTAL MYOCARDIAL INFARCTION IN THE DOG

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Recent work by Wroblewski and LaDue, and others has shown that serum glutamic oxaloacetic transaminase (GOT) (1, 4–6, 9–14, 16–18, 20, 22, 25, 26) and lactic dehydrogenase (12, 18, 24–26) activities are increased following clinical (4–6, 9–11, 14, 17, 20, 22, 25, 26) and experimental (1, 8, 12, 13, 16, 18, 24) myocardial infarction. Serum GOT has consequently become a standard diagnostic test of myocardial infarction. There is evidence that parallel to this increase in activities of enzymes in the serum, there is a loss of activities of the corresponding enzymes in the myocardium (8, 12, 16, 24). Can it be said, then, that extrusion of intracellular constituents, including enzymes, may occur from the necrotic cells?

Experimental myocardial infarction provides a method of studying enzymatic activities in both serum and heart muscle. Since the cardiac musculature is particularly rich in cytochrome *c* and cytochrome oxidase, it seemed to us of interest to determine whether following experimental myocardial infarction an increase in the activities of these two enzymes could be found in the serum.

Methods and Technique

Procedure

Mongrel dogs, 6 to 16 kg. in weight, were used for the experiments. As anesthetic, intraperitoneal Nembutal (30 mg. per kg.) was used. Respiration was maintained by means of insufflation through a tracheal cannula. In 16 dogs a high ligation of the anterior descending branch of the left coronary artery was performed. Two dogs died during the operation and two postoperatively of ventricular fibrillation. For the controls, sham operations were performed on two dogs, including tracheotomy and pericardiotomy, with the

placing of an untied ligature around the anterior descending branch of the left coronary artery.

Electrocardiograms were taken before, during, and at various intervals after the operations. Prior to or after ligation most animals received depot penicillin (daily), procaine amide and aminophylline (R) (15 mg. per kg.) intramuscularly. The concentrations of Nembutal (R) and other drugs used had no influence upon the activity of the enzymes, when studied *in vitro* and *in vivo*.

Venous blood samples were obtained at various intervals in several instances before anesthesia, in all cases before ligation; further samples were drawn 4, 8, 12, 24 and 48 hours after the coronary occlusion, and later at differing intervals.

After clotting, the blood was centrifuged at a low temperature and the activities of the enzymes estimated either immediately or after overnight storage in the refrigerator at 4° C.

The samples of the normal left and right ventricle, and the infarcted left ventricular area were removed immediately after death or sacrifice of the animal, and the enzyme activity estimated at once or after overnight freezing. In nine dogs, sections of the same areas as taken for enzymatic analysis were also studied histologically (J. Sinclair). In some instances the animals died within 48 hours; others were sacrificed for enzyme estimations in the heart at various intervals up to 22 days after infarction.

Chemical Methods

Transaminase Estimation

a) *Serum:* Method of Karmen, Wroblewski, and LaDue, (5) modified by Steinberg and Ostrow (22).

b) *Heart Muscle:* Extraction according to Cammarata and Cohen (2).

Estimation: Same as in a).

Units: Decrease of 0.001 opt. density per minute (t 25° C., d 1 cm) represents one unit. Units are calculated for 1 ml. serum and for 1 g. heart muscle (wet weight).

Lactic Dehydrogenase Estimation

a) *Serum:* Method of Wroblewski and LaDue (24).

b) *Heart Muscle:* Extraction according to Cammarata and Cohen (2).

Estimation: Same as in a).

The same homogenate is used as in the transaminase estimation. Units as in transaminase estimation.

Cytochrome c Estimation

a) *Serum:* Application of the method of Rosenthal and Drabkin (15). Four to six ml. of serum were used for each estimation.

b) *Heart Muscle:* Method of Rosenthal and Drabkin (15).

While transaminase, lactic dehydrogenase and cytochrome oxidase could be estimated on the same extract, the estimation of cytochrome *c* required a special procedure which had to start with a separate muscle sample.

Cytochrome Oxidase Estimation

a) *Serum:* Application of the method of Smith (21).

With a mixture of 0.5 ml. serum, 0.5 ml. reduced cytochrome *c* (90 μM) and 1.0 ml. phosphate buffer (pH 7.0, 0.01 M), only in a few hemolyzed

samples a slow, probably unspecific rate of oxidation could be measured.

b) *Heart Muscle:* Method of Smith (21).

Units: A calibration curve was established with a purified pig heart enzyme preparation. The decrease in optical density within the first 30 seconds of the reaction was used for the calculation. At the start a decrease in optical density of 0.001 within the first 30 seconds was set for 5 units. The units correspond to the change in optical density per minute per g. of wet tissue.

Results

Experimental animals

Serum values

The serum enzyme estimations of all experimental dogs are listed in Table 1.

Transaminase

The serum activities showed a maximum rise at 8 hours (range: 4 to 12 hours) after coronary ligation, and dropped to near-normal levels within 3 to 4 days (Figs. I and IV).

Lactic dehydrogenase

The serum activities of this enzyme likewise showed a maximum rise at 8 (4 to 12 hours). Reductions to normal were delayed in most cases. Secondary rises were also seen as late as 7 days after operation (Figs. II and IV).

Cytochrome c

The activity of this enzyme in the serum showed early rises at 4 to 8 hours, declining definitely at 12 hours and reaching normal values at 24

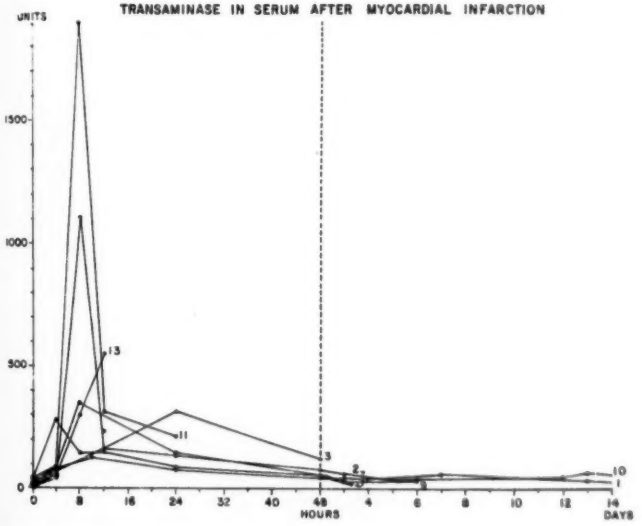

FIGURE I

TABLE 1

No.	Enzyme	Control	SERUM 4 hours	8 h	12 h	24 h	2 days	3 d	4 d
1	Transaminase	24 u.	84	134	(10 hrs.)	84	48	49	40
	Lact. Dehydrog.	415 u.	2,010	2,510	(10 hrs.)	2,200	1,005	1,600	2,500
	Cytochr. c.	0.00 mg%	0.04	0.04	(10 hrs.)	0.06	0.06	0.48	0.52
	Cytochr. o.	0.0 u.	0.0	0.0	(10 hrs.)	0.0	0.0	0.0	0.0
2	Tr.	22	54	350	235	148	51	22	
	L.D.	630	1,020	2,180	860	1,200	415	1,500	
	C.c.	0.05	0.09	0.11	0.04	0.05	0.02	0.00	
	C.o.	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
3	Tr.	22	....	....	....	319	125		
	L.D.	190	....	....	....	335	425		
	C.c.	0.03	....	....	....	0.07	0.06		
	C.o.	0.0	....	....	....	0.0	0.0		
4	Tr.	23	....	....	....	473	....	....	39
	L.D.	175	....	....	....	400	....	....	250
	C.c.	0.03	....	....	....	0.00	....	....	0.00
	C.o.	0.0	....	....	....	0.0	....	....	0.0
5	Tr.								
	L.D.								
	C.c.								
	C.o.								
6	Tr.	16	....	159					
	L.D.	490	....	1,170					
	C.c.	0.00	....	0.00					
	C.o.	....	....	....					
7	Tr.	17	106	(6 hrs.)	....	....	86		
	L.D.	280	680	(6 hrs.)	....	....	315		
	C.c.	0.00	0.19	(6 hrs.)	....	....			
	C.o.	....	....	....	....	....	....		
8	Tr.	34	67	(6 hrs.)	....	55			
	L.D.	765	1,050	(6 hrs.)	....	620			
	C.c.	0.08	0.04	(6 hrs.)	....	0.04			
	C.o.	....	....	....	....	....	....		
9	Tr.	37	283	149	160	92	61	35	....
	L.D.	900	3,420	2,260	3,285	2,900	3,150	1,370	....
	C.c.	0.02	0.16	0.82	0.57	0.09	0.00	0.02	....
	C.o.	0.0	....	0.0	36	0.0	0.0	0.0	....
10	Tr.	20	45	1,100	169	139	82	62	....
	L.D.	162	370	6,000	2,300	2,560	1,455	1,350	....
	C.c.	0.04	0.08	0.80	0.51	0.00	0.00	0.00	....
	C.o.	0.0	0.0	38	0.0	0.0	0.0	0.0	....
11	Tr.	39	90	1,940	315	220			
	L.D.	650	730	14,800	1,510	540			
	C.c.	0.00	0.575	0.46	0.53	0.11			
	C.o.	0.0	0.0	45	0.0	0.0			
12	Tr.	15	62						
	L.D.	322	373	(1 hr.)					
	C.c.	0.06	0.32						
	C.o.	....	....						
13	Tr.	17	71	302	551				
	L.D.	142	320	405	530				
	C.c.	0.03	0.30	0.49	0.47				
	C.o.	....	....	....	....				
14	Tr.	13	34	60	66	58			
	L.D.	100	145	253	320	80			
	C.c.	0.00	0.00	0.00	0.08	0.08	SHAM OPERATION		
	C.o.	0.0	0.0	0.0	0.0	0.0			
15	Tr.	37	41	79	97	90			
	L.D.	340	420	610	560	93	SHAM OPERATION		
	C.c.	0.00	0.16	0.16	0.24	0.24			
	C.o.	....	....	....	....	....			

TABLE 1

5 d	6 d	7 d	12 d	13 d	19 d	22 d	MYOCARDIUM Normal Right Ventr.	MYOCARDIUM Normal Left Ventr.	MYOCARDIUM Infarct
....	....	60	....	34	....	22	220x10³u	205x10³u	198x10³u
....	....	4,250	....	1,400	....	585	280x10³u	310x10³u	290x10³u
....	....	0.10	....	0.02	....	0.00	13.0mg%	13.1mg%	12.6mg%
....	....	0.0	....	0.0	....	0.0	1.06x10³u	1.23x10³u	0.95x10³u
							158	178	56
							293	330	84
							13.4	15.5	6.35
							1.69	1.72	0.86
							205	210	145
							233	250	102
							21.9	23.4	9.77
							0.92	0.95	0.53
							173	165	97
							300	310	140
							18.4	17.9	7.50
							1.01	1.01	0.47
							70	85	89
							210	345	100
							32.2	37.8	11.1
							1.34	1.40	1.12
							171	190	78
							230	227	170
							18.8	21.0	12.9
							0.98	1.07	0.55
							174	200	97
							270	280	140
							22.8	22.7	12.6
							1.07	0.95	0.48
							189	177	144
							339	340	224
							17.7	24.0	17.7
							0.81	1.24	0.72
....	43						192	190	180
....	2,000						276	249	323
....	0.03						...	...	...
....	0.0						1.27	1.57	1.26
37	45	....	48	67	32		...	163	200
2,200	3,850	....	3,380	3,000	2,100		...	230	250
0.00	0.02	....	0.05	0.43	0.16		...	15.5	16.4
0.0	0.0	....	0.0	0.0	0.0		...	1.74	2.2
							...	...	...
							...	...	...
							...	...	...
							...	...	...
							200	200	181
							222	195	230
							...	...	...
							0.75	0.95	0.75
							...	140	150
							...	61	219
							...	18.3	16.4
							...	...	4.45

hours in most cases. Secondary rises at 5 and 13 days postoperatively were noted in two dogs (Nos. 1 and 10, Figs. III and IV). In normal serum the values detected with this method ranged from traces to 0.08 mg%.

Cytochrome oxidase

Except for two sera (dogs 10 and 11) which were hemolyzed, no activity of this enzyme could be demonstrated before or after ligation.

Table 2 shows that the maximum increases (at 8 hours), as compared to the average initial values, were 27 times for transaminase, 16 times for cytochrome *c*, and 12 times for lactic dehydrogenase.

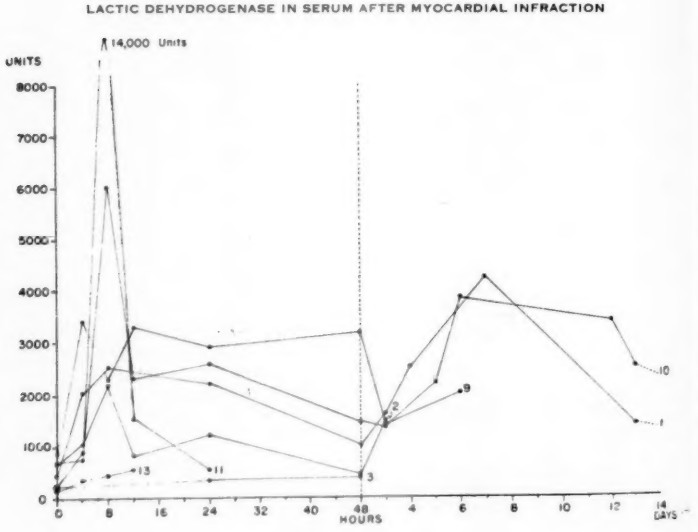

FIGURE II

TABLE 2

	Average Increase in Serum Enzyme Activities 8 Hours After Myocardial Infarction	Increase in Serum Enzyme Activities After Sham Operation	Average Decrease in Enzyme Activity in Infarcted Heart Muscle	Difference of Enzyme Activities in the Normal Right Ventricle Compared with Normal Left Ventricle
Transaminase	27 x normal	3–5 x normal	27%	–2.4%
Lact. Dehydrog.	12 x normal	2–3 x normal	29%	–9.8%
Cytochrome c	16 x normal	0–6 x normal	44%	–9.8%
Cytochrome Oxid.	.....	.....	29%	–9.7%

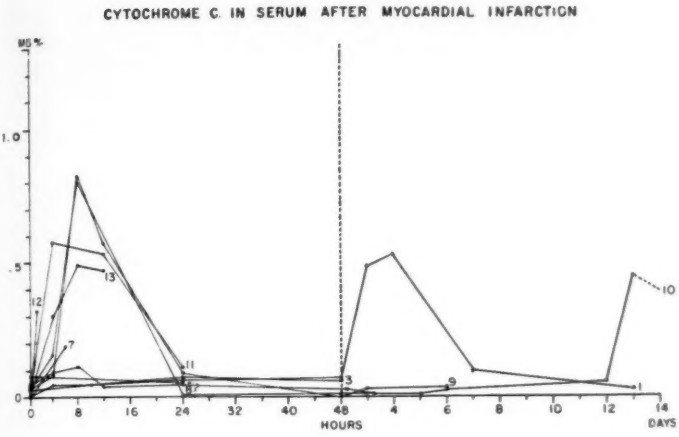

FIGURE III

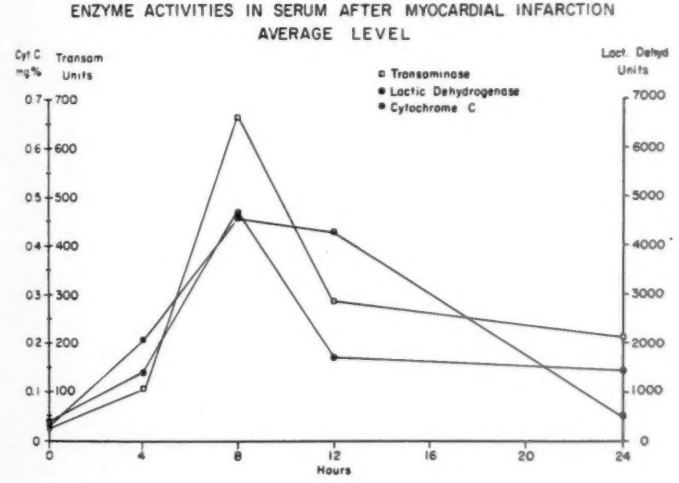

FIGURE IV

Myocardial Values

Table 1 lists the estimations of the four enzyme activities in normal and infarcted myocardium of the experimental animals. As controls served the right ventricular and the uninfarcted left ventricular muscle.

In most instances the musculature of the uninfarcted left ventricle showed

the highest activities of all the enzymes, exceeding those of the unaffected right ventricle in a consistent, though not statistically significant, fashion (Table 1). This variation is apparently more marked in regard to lactic dehydrogenase, cytochrome oxidase and cytochrome *c*. The infarcted areas of the left ventricle, patchy as they usually were, showed a general, but not constant, decrease in activity as compared with the controls. Average values are given in Table 1.

Table 2 shows the comparison between the drop of enzyme activities in heart muscle and the rise in the serum activities at 8 hours (the usual maximum). The average decrease in the heart muscle is similar for the transaminase, lactic dehydrogenase, and cytochrome oxidase, *i.e.*, 27 per cent to 29 per cent of the control activity of the left ventricle. The decrease in cytochrome *c* is the highest (44 per cent).

Pathological (J. G. Sinclair) and *Electrocardiographic* (A. Ruskin) *Data*

Dog No. 1. 22-day infarct: myocardial fibers swollen, granular, necrotic in large areas, with loss of striations; elsewhere—round nuclei swollen and lighter or more pyknotic than normal; dense or loose granulation tissue, predominant replacement fibrosis.

Liver: fatty infiltration; nuclei less dense or necrotic; central necrosis, obliteration of sinusoids.

ECG; Q_{12}, T_{12} negative, S-T_1 convex up, ventricular ectopics; paroxysmal ventricular fibrillation (reversed by Pronestyl (R)).

Dog No. 2. 76-hour infarct: foci of myocardial fibers swollen, necrotic, shrunken, or absorbed, and being replaced by fibrous tissue; cellular infiltration in parts.

Liver: Focal swelling, granulation, necrosis; nuclei dense and often eccentric; some fatty infiltration; hepatic and portal congestion with obliteration of sinusoids; hypertrophy of Kupffer cells.

ECG: Wide Q_1, T_1 negative, T_2 low, S-T_1 elevated, many ventricular ectopics.

Dog No. 3. 48-hour infarct: myocardial fibers shrinking, losing striations, or swollen; nuclei pyknotic and being ejected by surrounding vacuolations; beginning infiltration with large round cells.

ECG: Q_{12} (as before), T_{123} negative, S-T_{12} elevated (as before), ventricular ectopics, paroxymal ventricular tachycardia.

Dog No. 4. 96-hour infarct: myocardial fibers undergoing various stages of swelling, shrinkage, hyalinization, removal, with many pale ghost areas; heavy infiltration of monocytes and polymorphonuclears; obliteration of capillaries, fibroblasts proliferating actively, with prominent nuclei and numerous mitoses, replacement fibrosis.

ECG: Q_{12} (as before) T_{12} negative, T_3 higher, S-T_{23} elevated, frequent ventricular ectopics.

Dog No. 6. 9-day infarct: alternate areas of swollen, shrunken, or absent myocardial fibers, with predominant replacement fibrosis.

ECG: Q_{12}, T_{12} negative, S-T_1 elevated.

Dog No. 7. 48-hour infarct: myocardial fibers swollen, nearly obliterating the capillaries; nuclei and striations mostly lost; areas of smaller shrunken fibers infiltrated with polymorphonuclears and mononuclear cells, arterial endothelium swollen.

Liver: small foci of swelling and granulation; otherwise fairly normal.

ECG: Q_1, T_1 negative (as before), T_2 negative, S-T_{12} elevated, a few ventricular ectopics.

Dog No. 8. 6-day infarct: myocardial fibers show marked degenerative necrosis, with infiltration of mononuclear and polymorphonuclear cells; early fibroblastic (*e.g.*, nuclear) proliferation with complete fibrosis in places.

ECG: Q_{12} (as before), S-T_1 elevated, T_{12} negative (as before).

Dog No. 9. 6-day infarct: several areas of focal necrosis with apparent calcification in spots; no cellular infiltration or fibrosis.

ECG: Q_{12}, T_{12} negative, S-T_1 convex up, ventricular ectopics.

Dog No. 11. 40-hour infarct: indistinct striations and fading nuclei of myocardial cells; infiltration with monocytes and some polymorphonuclears; no fibrosis.

ECG: S-T_{12} elevated, T_1 negative, T_2 low, few ventricular ectopics only.

Microscopic summary

The most striking feature of the sections was the lack of uniformity. Fiber swelling was followed by sharply marked areas of shrinkage and nuclear ejection (dogs 2, 3, 9, 11). Monocytic, then polymorphonuclear, leucocytic infiltration (dogs 2, 4, 7, 8, 11) from the epicardial (and endocardial) surfaces, with sharply marked fronts, occurred among the hyalinized, shrunken fibers only. Hyalinization and dissolution followed only in these infiltrated areas, and here only were fibroblastic proliferation with striking mitoses seen (dogs 4, 8). Replacement fibrosis was not complete until the leucocytes had disintegrated and disappeared (dogs 1, 6, 8).

Electrocardiographic summary

Classical electocardiographic findings of S-T elevations, Q waves, and T negativities in leads I and II generally occurred at various intervals after ligation of the descending branch of the left coronary artery. Ventricular ectopics and even paroxysmal ventricular fibrillation were also frequently recorded. Aminophylline(R) and Pronestyl(R) had variable effects in reversing these changes.

Control Animals

Sera of dogs 14 and 15, which had sham operations, showed slight rises in the enzyme activities, as seen in table 1. The max-

imum activities of the enzymes (8 to 12 hours after surgical intervention) were: for transaminase 2 to 5 times normal, for the lactic dehydrogenase 2 to 3 times normal, for cytochrome *c* up to 0.08–0.24 mg. per cent.

Discussion

Serum Values

Our results confirm previous reports of the rise in serum transaminase and lactic dehydrogenase activities after experimental myocardial infarction. Furthermore, it was found that cytochrome *c* activity rises in the serum under the same circumstances. We used the method of Rosenthal and Drabkin (15), as the most sensitive one available, yet in normal serum the cytochrome *c* was often undemonstrable. Moreover, in samples with inadvertent hemolysis, traces of blood pigments may interfere in non-specific fashion with its determination.

Available methods for cytochrome oxidase are in our experience not sensitive enough for demonstration of its activity in normal serum; nor were elevations found after infarction, except in two blood samples with a high degree of hemolysis (see Chemical Methods). In addition, the failure to demonstrate this enzyme in the serum may be, in part, due to its suspected strong mitochondrial fixation and relative insolubility as contrasted with the other enzymes under discussion.

It must be emphasized that in our studies, all three enzyme activities rose and fell in the serum approximately at the same time after the production of the infarct (Fig. IV)—generally at the very onset of the pathological changes.[8] The lactic dehydrogenase activity did not rise earlier (9) or later (26) than serum GOT, as claimed by some workers. On the other hand, lactic dehydrogenase (Table 1, dogs 1, 9, 10) and cytochrome *c* (dogs 1, 10) showed secondary rises (*cf.* Zimmermann *et al.* (26)) many days (up to 13) after infarction. These may be explained, perhaps, on the basis of secondary infection (empyema, pneumonia), reacting probably on the liver. In this connection, even those animals showing microscopic evidences of liver involvement (dog 1—fatty liver; dogs 2, 7, 10) showed negative serum thymol turbidity and cephalin flocculation test. It may be that the slightest involvement of the liver, and possibly of other organs and tissues very rich in lactic dehydrogenase and cytochrome *c*

activity, causes release of these enzymes into the serum. For diagnostic reasons, the greater rise of transaminase, as well as its higher specificity, would suggest its estimation to be preferable. It rose, in general, higher in massive infarcts (dogs 1, 2—both infarcts greater than 3 cm^3).

Chest operations (1) and other types of skeletal muscle damage (23) have been found to elevate transaminase activity, but the rise has not exceeded 5 to 6 times the control values (up to 180 units of serum GOT). Values obtained by us are lower (66 and 97 units, Table 1); nor do lactic dehydrogenase or cytochrome *c* activities reach values any higher than 3 to 6 times the original levels in the control dogs. All these values are much lower than those in the experimental animals.

Myocardial values

Previous studies of enzyme activities in infarcted cardiac muscle have shown falls in transaminase (8, 12, 13), lactic dehydrogenase, (26) and diphosphopyridine nucleotide. (3) The latter and cytochrome *c* also decrease (17 and 30 per cent respectively) in anoxic heart muscle. (7)

The drop in transaminase, lactic dehydrogenase, and cytochrome oxidase activity in the infarcted muscle to about 30 per cent, is comparable to the 31 per cent fall of transaminase reported by Lemley-Stone *et al.* (8) These facts, per se, merely indicate that in a necrotic cell, enzymatically-active proteins undergo a lytic process. The marked drops (down to 5–10 per cent) in transaminase activity in the older infarcts reported by Agress *et al.* (1) may have been due to their larger sizes (necrosis, fibrosis, etc.) produced by the coronary embolism. We should emphasize again that meticulous care in the choice of truly infarcted areas highly influences the results.

The greater decrease of the cytochrome *c* content (44 per cent) is not significantly different from that of the other enzymes. It could have been influenced by the fact that we had to use a different muscle sample for cytochrome *c* estimation, whereas the three other enzymes were analyzed from one original homogenate.

Histological evidences of myocardial necrosis, cellular infiltration, and fibrosis were seen in various degrees in all animals demonstrating decreased enzyme activities within the infarcted

areas (dogs 2, 3, 4, 6, 7, 8, 11; see *Pathological Data*). No definite parallelism, however, was found between the actual degree (and duration) of the myocardial damage and the fall in enzyme activities in the infarcted areas. It is true that dog 11, showing minimal necrosis and cellular infiltration, had very slight drops in transaminase and lactic dehydrogenase activities in the involved area. We cannot explain definitely, on the other hand, the minimal decrease in all four enzyme activities in the massive, older infarct of dog 1. Other workers, having produced, in general, larger infarcts did find relations. (1, 8)

As to the slightly higher enzyme activities in normal left (as compared to right) ventricle, the following considerations are pertinent. Since the greater musculature and work of the left ventricle may not involve any higher content of protein per gram of muscle, the relatively higher enzyme activities could be an adaptive response of higher enzyme production to the higher (work and) metabolic demands. In this connection, it has been shown that adenosinetriphosphate and succinic dehydrogenase (19) have also been found at higher levels of activity in the left than in the right ventricle.

If we now compare the serum and myocardial values of the enzyme activities, we find generally inverse relations in the rises in the one and the falls in the other medium respectively. Dogs 1 and 2 seem to be apparent exceptions. In the case of dog 1, the two possible explanations would be poor selection of material or regeneration of enzymes after 22 days. In the case of dog 2, the enormous rise in serum enzyme activities of this earliest studied infarct (see *Pathological Data*) was evidently not reflected in either chemical or histological tissue alterations.

Summary and Conclusions

1. Experimental myocardial infarction was produced in 16 dogs by ligation of the anterior descending branch of the left coronary artery.

2. Glutamic-oxaloacetic transaminase, lactic dehydrogenase, cytochrome *c*, and cytochrome oxidase estimations were done in the serum and the infarcted and normal parts of the myocardium.

3. Elevations of serum activities of all enzymes investigated except cytochrome oxidase were most marked at 8 hours following infarction. The degrees of elevation were: transaminase 27x, cytochrome *c* 16x, and lactic dehydrogenase 12x. In contrast, sham-operated dogs showed only slight rises (2 to 6x) in the activities of these enzymes.

4. Decreases in the activities of all four enzymes (27–44 per cent) were seen in infarcted muscle as compared with normal areas of the left ventricle.

5. No exact parallels could be drawn between the cytological and chemical changes, except that serum enzyme activities were highest at the onset of the pathological alterations.

6. Normal left ventricle showed slightly higher activities of all four enzymes than normal right ventricle.

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[g] It is relevant that the striking elevations noted in other studies were: for serum aldolase, malic dehydrogenase, isomerase activities— 24 hours; serum globulins and C-reactive protein—third day; temperature, white blood count, sedimentation rate—second day; urine urobilinogen, coproporphyrins—first two or three days; 11-oxycorticoids and 11-ketosteroids—third to fifth day; but serum postassium, lactate, pyruvate—almost immediately after coronary occlusion.

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SMOKING AND DISEASE
A STUDY BASED UPON 12,050 INDIVIDUALS

R. H. Rigdon and Helen Kirchoff[a]

Cutler, a statistician, stated in June of 1955, "There is disagreement whether the evidence at hand warrants a conclusion that smoking and lung cancer are causally related . . . that, if the evidence pointing to a real increase in lung cancer mortality is accepted, one is led to the hypothesis that it is probably due to environmental factors. . . . A number of environmental changes, not mutually exclusive, have been suggested as causal factors in the increase of lung cancer mortality. These are: (1) increased use of cigarettes, (2) increased atmospheric pollution by motor vehicle exhausts, factory wastes, etc., and (3) increased occupational exposure to known cancer producing substances." In his summary of the findings of fourteen retrospective studies, reported since 1939 and showing an association between smoking and lung cancer, Cutler showed that the estimate of relative risk for developing lung cancer as compared to nonsmokers varied from 1.2 to 36.4 for all smokers and from 1.9 to 79.0 for heavy smokers.

Recently we studied the smoking habits of 21,612 individuals in Texas 15 years of age and older (Kirchoff and Rigdon, 1956). This study showed that although there was no significant difference between the percentage of smokers in the group of white males and colored males, or in the white females and colored females, there was a variation in the amount of cigarettes smoked. Sixty-three and one-tenth per cent of the white males and 33.4 per cent of the white females were present smokers. Fifty-three per cent of the white and 43 per cent of the colored male present cigarette smokers smoked an average of a pack of cigarettes a day. Of the white males who were present cigarette smokers, 5.5 per cent had smoked two packs or more daily for 16 years or longer. The corresponding figure for the colored male was 2.0 per cent. Of all males 40 to 49 years of age, 72.0 per cent were present cigarette smokers; of this group, 79.0 per cent smoked a pack or more daily and 74.5 per cent had smoked for 16 years

or longer. In this study there were 12,050 individuals who were either hospital or clinic patients at the time the survey was made. The medical diagnosis, as given on the hospital records, was obtained and correlated with their smoking history. The results are reported at this time.

Methods

The techniques used in making this smoking survey have been discussed in two previous publications (Kirchoff and Rigdon, 1954 and 1956). The diagnoses were taken directly from the hospital records. When the hospital charts listed more than one diagnosis, the primary disease process was used. For instance, if a patient was being treated in the clinic for syphilis, but on the day of the interview had a diagnosis of acute sinusitis, he was classified under the diagnosis of syphilis for the purpose of this study. Since none of these individuals either came to the clinic or were hospitalized because they smoked, it is felt that a correlation of the primary disease with the smoking history would not constitute a selection of a group on the basis of smoking. Many of the lung cancer patients were referred to John Sealy Hospital from all over Texas for specific diagnosis and treatment, so the number of such patients in the hospital would vary from week to week and month to month. This factor must be considered in evaluating the frequency of this lesion. The problem of patients with bronchogenic carcinoma being concentrated in diagnostic centers within the state has been discussed (Rigdon *et al.*, 1955).

Diseases were coded for the IBM punch cards according to the classification given in the Standard Nomenclature of Diseases. Many of the diagnoses were then combined. Twenty-four categories were used for correlation with smoking habits. Most of the disease categories are self-explanatory. Infectious disease included bacterial, parasitic and viral agents. The group of miscellaneous diseases comprised a wide variety of lesions which we thought would not be considered by anyone to be associated with smoking. The number of individuals in some of the groups is too few to be considered statistically; however, it is recorded.

The smoking data were separated according to sex, race and age. Three age groups, 20 to 39 years, 40 to 59 years, and 60 years and over, were used in comparing disease processes with smoking habits. The age group 15 to 19 years was not included because the majority of these individuals had smoked 5 years or less. The smoking data were based on both the present and past smokers (cigarettes, pipes and/or cigars), present and past cigarette smokers, and the amount and length of time in years that the present cigarette smokers had smoked. In these data some of the cigarette smokers also smoked a pipe and/or cigars; however, for this study only the cigarette smoking was used. Pipes and cigars were not converted to cigarettes. A present smoker was one who currently smoked or had smoked within the past eleven months. A past smoker was one who had not smoked for a year or longer. A nonsmoker was one who had never smoked or had smoked only one or two cigarettes a month for less than a year or only an occasional cigar or pipe. The number of cigarettes smoked per day was grouped as follows:

	cigarettes
1. Less than five cigarettes	
2. One-half pack	5–14
3. One pack	15–24
4. One and one-half packs	25–34
5. Two packs	35–44
6. More than two packs	45+

Table 1 gives the percentage of present, past and nonsmokers according to age, sex and race. Seventy-two and four-tenths per cent of all white males and 73.1 per cent of all colored males were present smokers (all ages, adjusted using population in the United States 15 years of age and older for 1950). There were 34.1 per cent smokers among the white females and 31.2 per cent among the colored females. In the older age groups there was a decrease in the percentage of present smokers and an increase in the percentage of past smokers among the males.

The number interviewed and the percentage of present and past cigarette smokers by age, sex and race are given in table 2. The average percentage of present white male cigarette smokers between the ages of 20 and 59 was 75.2. The percentage of colored male cigarette smokers was slightly less during this period of life. Sixty-seven and eight-tenths per cent of the white males and 34.0 per cent of the white females were present cigarette smokers in this survey. Seven and two-tenths per cent of the white males and 5.2 per cent of the colored males were past cigarette smokers. Of the white male present cigarette smokers, 5.5 per cent also smoked a pipe, 4.9 per cent cigars,

TABLE 1

PERCENT DISTRIBUTION OF PRESENT, PAST AND NONSMOKERS ACCORDING TO AGE, SEX AND RACE

AGE GROUP	PRESENT				PAST				NONSMOKERS			
	WM	CM	WF	CF	WM	CM	WF	CF	WM	CM	WF	CF
15-19	40.3	48.3	29.6	20.0	1.6	-	1.2	2.7	58.1	51.7	69.2	77.3
20-24	72.3	73.3	41.7	38.1	3.4	8.3	3.1	1.2	24.3	18.3	55.1	60.7
25-29	77.2	83.9	49.2	42.2	3.9	2.1	4.4	2.5	18.9	14.0	46.4	55.3
30-39	81.2	81.2	46.9	40.8	8.6	5.3	4.5	6.4	10.2	13.5	48.6	52.8
40-49	81.7	77.9	36.0	30.4	8.0	5.0	6.1	4.1	10.3	17.1	57.9	65.5
50-59	78.5	73.7	24.6	22.7	9.9	9.0	3.7	6.7	11.6	17.3	71.7	70.6
60-69	65.8	69.4	15.0	17.4	18.2	18.1	3.6	9.2	16.0	12.5	81.4	73.4
70+	55.2	56.7	10.1	9.6	21.6	22.8	1.4	4.8	23.2	20.5	88.5	85.6
All ages	71.8	71.4	34.3	32.3	11.3	10.5	4.0	4.3	16.9	18.1	61.7	63.4
Adjusted age*	72.4	73.1	34.1	31.2	8.8	6.8	3.9	4.6	18.1	20.1	62.0	54.2

*Adjusted for age using population of the United States for 1950 for sex and race.

TABLE 1. Shows per cent distribution of present, past and nonsmokers according to age, sex and race. All ages, in this and subsequent tables, adjusted using population of the United States 15 years of age and over for 1950 for that sex and race.

TABLE 2

NUMBER INTERVIEWED AND PERCENTAGE OF PRESENT AND PAST CIGARETTE SMOKERS BY AGE, SEX AND RACE

AGE GROUP	WHITE MALES			COLORED MALES			WHITE FEMALES			COLORED FEMALES		
	No.	Present	Past	No.	Present	Past	No.	Present	Past	No.	Present	Past
15-19	191	39.8	1.6	87	46.0	-	240	29.6	1.2	300	20.0	2.7
20-24	148	71.6	3.4	60	68.3	8.3	448	41.7	3.1	522	37.9	1.1
25-29	206	74.8	3.9	93	82.8	1.1	435	49.2	4.4	483	42.0	2.5
30-39	499	78.8	7.8	208	76.0	5.3	817	46.9	4.5	627	40.8	6.4
40-49	690	77.5	7.4	280	66.8	3.6	720	36.0	6.0	487	29.8	3.9
50-59	729	71.3	8.5	289	58.1	6.2	566	24.6	3.5	313	21.7	6.1
60-69	647	58.7	13.3	281	53.0	11.0	414	14.7	3.6	207	13.5	6.3
70+	458	36.0	11.6	224	30.8	13.8	277	9.4	1.1	104	4.8	2.9
All ages	3,568	65.3	8.6	1,522	58.4	7.0	3,917	34.2	3.9	3,043	31.6	3.9
Adjusted age*		67.8	7.2		64.5	5.2		34.0	3.8		30.4	4.2

*Adjusted for age using population of the United States for 1950 for sex and race.

TABLE 2. Shows number interviewed and percentage of present and past cigarette smokers by age, sex and race.

and 1.6 per cent all three; of the colored male present cigarette smokers, 7.8 per cent smoked a pipe, 7.8 per cent cigars and 2.6 per cent all three. Only 3.8 per cent of the white and 4.2 per cent of the colored females were past cigarette smokers. The percentage of past smokers (cigarettes, pipes and/or cigars) in the white males was 8.8 and 6.8 for the colored males; the corresponding figures for the white and colored females were 3.9 and 4.6 per cent, as shown in table 1.

The average amount of cigarettes smoked by the 5,521 present cigarette smokers according to age, race and sex is given in table 3. Fifty per cent of the white males who were present cigarette smokers, 55.5 per cent of the colored males, 37.8 per cent of the white females, and 28.0 per cent of the colored females smoked one pack of cigarettes a day. Twenty-three per cent of the white males, 9.1 per cent of the colored males, 8.6 per cent of the white females and 3.2 per cent of the colored females smoked more than a pack of cigarettes a day. Table 4 gives the length of time the different groups have smoked cigarettes.

The length of time and the number of cigarettes smoked daily by the 5,521 present cigarette smokers is shown in table 5. Of the white males who smoked cigarettes, 39.0 per cent have smoked a pack a day, 10.6 per cent have smoked one and one-half packs and 9.0 per cent have smoked two or more packs a day for 16 years or longer. Corresponding figures for the colored male are 36.9 per cent, 4.6 per cent, and 2.6 per cent.

The individuals in this survey were divided into three age groups, 20 to 39 years, 40 to 59 years and 60 years of age and over, for correlating the different diseases with cigarette smoking habits. These data are given for the males in table 6 and for the females in table 7. There were two white males with cancer of the lung between the ages of 20 and 39 years; one of these smoked cigarettes. There were 33 white males with lung cancer in the 40 to 59 age group and 97 per cent smoked cigarettes. There were 11 colored males with lung cancer in the same group and 91 per cent were cigarette smokers. There were 29 white males with lung cancer 60 years of age and over and 59 per cent of these were present cigarette smokers and 21 per cent past cigarette smokers, giving a total of 80 per cent present and past cigarette smokers. There were 12 cases of cancer of the lung in colored males 60 years of age and over; of these 67 per cent were present cigarette smokers and 8 per cent past cigarette smokers.

In the group of colored males with tuberculosis there were 19 between the ages of 20 and 39 years; 100 per cent of these smoked cigarettes. There were 15 colored tuberculosis patients between the ages of 40 and 59 years and 100 per cent were either present or past cigarette smokers. In the group 60 years of age and older there were only six colored men; 50 per cent of these were present cigarette smokers and 17 per cent were past cigarette smokers. Of all white males between the ages of 20 and 39 years, regardless of their medical diagnosis, 77 per cent were present cigarette smokers and 6 per cent were past cigarette smokers; in the age group 40 to 59 years, 74 per cent were present cigarette smokers and 8 per cent were past cigarette smokers, and in the age group 60 years of age and over only 49 per cent were present cigarette

TABLE 3

AVERAGE DAILY AMOUNT OF CIGARETTES SMOKED BY 5,521 PRESENT CIGARETTE SMOKERS, BY AGE, RACE AND SEX

Age Group	Number of present cigarette smokers				Average amount of cigarettes smoked daily											
					< 1 Pack				1 Pack				> 1 Pack			
	WM	CM	WF	CF	WM	CM	WF	CF	WM	CM	WF	CF	WM	CM	WF	CF
15-19	76	40	71	60	57.9	62.5	63.4	85.0	34.2	35.0	28.2	13.3	7.9	2.5	8.4	1.7
20-24	106	41	187	198	27.4	31.7	53.5	71.2	51.9	58.5	38.0	24.7	20.7	9.8	8.5	4.0
25-29	154	77	214	203	23.4	40.3	44.4	69.9	53.2	53.2	47.2	28.6	23.4	6.5	8.4	1.5
30-39	393	158	383	256	20.6	32.3	43.3	62.5	51.7	57.0	46.2	34.4	27.7	10.7	10.4	3.1
40-49	535	187	259	145	15.7	33.7	51.3	62.1	55.3	52.9	39.4	34.5	29.0	13.4	9.3	3.4
50-59	520	168	139	68	19.0	41.7	56.1	64.7	54.4	49.4	35.3	33.8	26.5	8.9	8.6	1.5
60-69	380	149	61	28	32.6	47.0	70.5	71.4	47.4	42.3	24.6	17.9	20.0	10.7	4.9	10.7
70+	165	69	26	5	46.1	53.6	61.5	80.0	40.0	42.0	30.8	20.0	13.9	4.3	7.7	-
All ages	2,329	889	1,340	963	24.6	40.5	50.4	67.7	51.1	49.8	40.5	29.3	24.3	9.7	9.0	3.0
Adjusted age*					26.9	40.4	53.6	68.8	50.0	55.5	37.8	28.0	23.0	9.1	8.6	3.2

* Adjusted for age using population of the United States for 1950 for sex and race.

TABLE 3. Shows number of present cigarette smokers and average daily amount of cigarettes smoked by 5,521 present cigarette smokers, by age, race and sex.

TABLE 4

PERCENT DISTRIBUTION OF PRESENT CIGARETTE SMOKERS
ACCORDING TO THE NUMBER OF YEARS THEY HAVE SMOKED, BY AGE, RACE AND SEX

AGE GROUP	5 years or less				6 - 10 years				11 - 15 years				16 years or more			
	WM	CM	WF	CF	WM	CM	WF	CF	WM	CM	WF	CF	WM	CM	WF	CF
15-19	90.8	82.5	93.0	90.0	9.2	12.5	5.6	10.0	-	5.0	1.4	-	-	-	-	-
20-24	44.3	58.5	70.1	76.3	43.4	26.8	26.7	20.7	11.3	14.6	3.2	2.5	1.0	-	-	0.5
25-29	9.7	11.7	28.5	50.7	52.6	42.9	49.1	34.5	27.3	36.4	20.5	12.8	10.4	9.1	1.9	2.0
30-39	6.1	5.1	19.6	26.2	10.9	12.6	22.4	27.3	28.0	26.6	23.5	21.9	55.0	55.7	34.5	24.6
40-49	1.7	4.3	23.2	22.1	1.5	2.7	20.1	14.5	4.3	3.7	12.7	17.9	92.5	89.3	44.0	45.5
50-59	2.1	3.0	18.7	10.3	2.5	2.4	10.8	14.7	1.5	7.1	14.4	7.4	93.9	87.5	56.1	67.6
60-69	3.4	4.7	18.0	25.0	1.6	2.7	14.8	10.7	0.5	2.7	1.6	-	94.5	89.9	65.6	64.3
70+	2.4	4.3	3.8	-	1.2	2.9	11.5	20.0	1.8	1.4	11.5	-	94.5	91.3	73.1	80.0
All ages	8.2	10.9	32.1	43.7	8.8	9.4	24.2	23.0	8.6	11.5	14.8	12.3	74.3	68.2	28.9	21.0
Adjusted age*	16.6	20.9	31.7	39.4	14.1	13.6	20.6	20.1	11.0	14.2	12.8	10.8	58.2	51.3	34.9	29.7

*Adjusted for age using population of the United States for 1950 for sex and race.

TABLE 4. Shows per cent distribution of present cigarette smokers according to the number of years they have smoked, by age, race and sex.

smokers and 13 per cent past cigarette smokers. Corresponding percentages for the white female are much less.

Graphs I and II show the percentage of males 40 years of age and older who are present cigarette smokers, past cigarette smokers and nonsmokers in each of the disease categories. These graphs also show the percentage of present cigarette smokers who smoke less than one pack, one pack and more than one pack daily. The amount of cigarette smoking is less in the colored male than in the white male. The percentage of white male present cigarette smokers is approximately the same in the group with cancer of the lung, cancer of the larynx, tuberculosis, and even appendicitis. Of the white males with lung cancer 88.7 per cent are either present or past cigarette smokers. Of those with tuberculosis 90.1 per cent are either present or past cigarette smokers, while 94.7 per cent of the group with cancer of the larynx are either present or past cigarette smokers. Eighty-two and six-tenths per cent of the colored males with lung cancer are either present or past cigarette smokers and 90.5 per cent of those with tuberculosis are either present or past cigarette smokers.

Discussion

It is obvious from this survey that cigarette smoking was frequent in the majority of the white and colored male clinic and hospital patients interviewed between 1952 and 1954. In reviewing the data on the smoking habits of the male there occurs a marked variation in the habit of smoking in the different age groups. There also is a wide variation in the frequency of present

TABLE 5

NUMERICAL AND PERCENT DISTRIBUTION OF 5,521 PRESENT CIGARETTE SMOKERS ACCORDING TO NUMBER OF YEARS AND QUANTITY SMOKED DAILY, BY RACE AND SEX

Amt. of Cigarettes	No. Years Smoked	WM		CM		WF		CF	
		No.	%	No.	%	No.	%	No.	%
1/2 Pack	5 or less	111	4.8	62	7.0	306	22.8	337	35.0
	6-10	57	2.4	39	4.4	148	11.1	140	14.6
	11-15	36	1.5	45	5.1	84	6.3	65	6.8
	16 or more	369	15.8	214	24.1	138	10.3	110	11.4
1 Pack	5 or less	64	2.7	30	3.4	107	8.0	76	7.9
	6-10	109	4.7	40	4.5	147	11.0	75	7.8
	11-15	110	4.7	45	5.1	87	6.5	47	4.9
	16 or more	908	39.0	328	36.9	202	15.1	84	8.7
1 1/2 Pack	5 or less	12	0.5	2	0.2	11	0.8	4	0.4
	6-10	22	1.0	4	0.4	18	1.3	3	0.3
	11-15	26	1.1	7	0.8	21	1.6	4	0.4
	16 or more	246	10.6	41	4.6	31	2.3	7	0.7
2 Packs or more	5 or less	5	0.2	3	0.3	7	0.5	4	0.4
	6-10	18	0.8	1	0.1	11	0.8	4	0.4
	11-15	28	1.2	5	0.5	6	0.4	2	0.2
	16 or more	208	9.0	23	2.6	16	1.2	1	0.1
		2,329	100.0	889	100.0	1,340	100.0	963	100.0

TABLE 5. Shows numerical and per cent distribution of 5,521 present cigarette smokers according to number of years and quantity smoked daily, by race and sex.

TABLE 6

NUMBER OF MALES AND PERCENTAGE OF PRESENT AND PAST CIGARETTE SMOKERS IN EACH DIAGNOSIS ACCORDING TO AGE AND RACE

DIAGNOSES	20-39 years of age						40-59 years of age						60 years of age and over					
	Number of Cases		Percent Cigarette smokers				Number of Cases		Percent Cigarette smokers				Number of Cases		Percent Cigarette smokers			
			Present		Past				Present		Past				Present		Past	
	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C
Cancer																		
Lung	2	0	50	-	-	-	33	11	97	91	-	-	29	12	59	67	21	8
Larynx	0	0	-	-	-	-	9	2	89	50	11	-	10	1	70	100	20	-
Buccal Cavity	1	0	100	-	-	-	15	2	73	50	13	-	12	3	50	67	-	-
Other	12	2	75	100	25	-	116	33	75	67	10	-	162	57	44	47	10	14
Heart Disease																		
Hypertensive	18	8	78	62	-	13	83	41	72	59	10	12	65	48	48	29	11	15
Arteriosclerotic	2	2	100	100	-	-	46	18	78	72	9	6	92	41	45	46	21	15
Rheumatic	5	6	60	67	40	33	12	3	75	67	17	33	2	0	50	-	-	-
Lymphoma	3	2	67	50	-	-	9	4	44	50	11	-	13	4	69	-	8	25
Brain Tumors	5	1	80	100	-	-	9	1	56	100	-	-	2	0	-	-	50	-
Benign Tumors	9	5	67	40	-	-	14	6	86	67	-	-	12	4	75	50	-	-
Infectious diseases	20	6	75	100	5	-	12	10	75	40	-	-	6	3	50	33	-	33
Syphilis	10	21	80	81	-	5	36	62	75	60	6	8	16	37	62	57	6	8
Tuberculosis	70	19	81	100	6	-	65	15	82	93	8	7	16	6	75	50	19	17
Respiratory diseases	59	23	73	74	2	9	91	25	81	80	8	-	51	16	67	38	14	13
Appendicitis	16	7	75	86	-	-	5	4	80	50	-	-	0	1	-	100	-	-
Diseases of G. U. and G. I. Tract	113	35	80	74	7	9	214	91	75	58	7	3	198	112	46	46	13	12
Diseases Bone and Joint	45	9	84	89	2	-	62	15	71	53	13	13	41	19	63	21	5	-
Allergic disorders	10	13	50	54	20	8	21	3	71	67	10	-	19	2	47	100	32	-
Thyroid disorders	11	2	55	100	9	-	15	6	67	50	7	17	5	0	60	-	40	-
Diabetes	16	5	81	80	-	-	39	15	59	53	10	-	35	21	34	19	11	19
Trauma	121	79	85	76	3	1	96	50	79	70	3	-	25	9	60	89	8	-
Hernia	21	12	71	67	10	17	43	14	74	71	5	-	44	19	46	42	14	21
Miscellaneous	284	104	73	76	8	4	374	138	70	57	9	7	250	90	47	40	11	12
ALL DIAGNOSES	853	361	77	77	6	5	1419	569	74	62	8	5	1105	505	49	43	13	12

TABLE 6. Shows the number of males and percentage of present and past cigarette smokers in each diagnosis, according to age and race.

TABLE 7

NUMBER OF FEMALES AND PERCENTAGE OF PRESENT AND PAST CIGARETTE SMOKERS IN EACH DIAGNOSIS

ALL DIAGNOSES	853	361	77	77	6	5	1419	569	74	62	8	5	1105	505	49	43	13	12

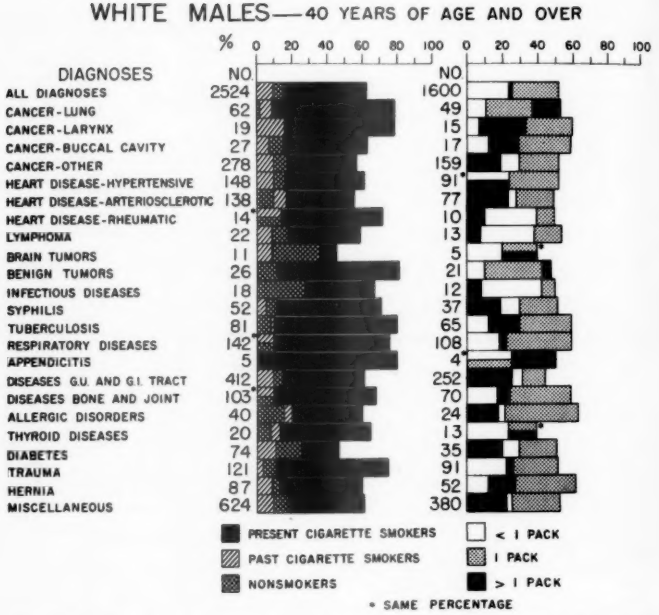
TABLE 6. Shows the number of males and percentage of present and past cigarette smokers in each diagnosis, according to age and race.

TABLE 7

NUMBER OF FEMALES AND PERCENTAGE OF PRESENT AND PAST CIGARETTE SMOKERS IN EACH DIAGNOSIS ACCORDING TO AGE AND RACE

DIAGNOSES	20-39 years of age						40-59 years of age						60 years of age and over					
	Number of Cases		Percent Cigarette smokers				Number of Cases		Percent Cigarette smokers				Number of Cases		Present Cigarette smokers			
			Present		Past				Present		Past							
	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C
Cancer																		
Lung	0	0	-	-	-	-	6	1	50	100	17	-	3	0	33	-	33	-
Larynx	0	0	-	-	-	-	2	0	-	-	-	-	0	1	-	-	-	-
Buccal Cavity	0	1	-	-	-	-	4	0	50	-	-	-	2	0	50	-	-	-
Other	41	22	46	36	7	-	102	51	28	20	10	6	76	31	13	7	4	3
Heart Disease																		
Hypertensive	30	24	33	38	10	4	75	122	27	23	4	4	60	63	17	10	2	6
Arteriosclerotic	0	4	-	-	-	25	26	11	35	9	4	18	59	17	15	12	-	6
Rheumatic	14	6	36	33	-	-	11	2	46	-	-	-	2	0	-	-	-	-
Lymphoma	4	2	25	-	-	-	4	2	25	50	-	-	4	2	-	-	-	50
Brain Tumors	3	4	33	50	-	-	7	1	-	100	-	-	5	0	-	-	-	-
Benign Tumors	19	7	37	29	5	-	20	8	15	-	10	-	10	8	10	13	10	-
Infectious diseases	16	13	38	46	-	-	5	5	60	40	-	-	3	2	-	50	-	-
Syphilis	14	69	71	52	7	6	16	51	38	37	-	2	8	14	25	36	13	-
Tuberculosis	31	15	48	40	3	-	10	3	20	67	20	-	4	3	25	-	-	-
Respiratory disease	57	38	63	45	4	-	39	30	49	33	8	10	22	11	5	9	9	-
Appendicitis	4	2	75	-	-	-	2	1	-	100	100	-	1	0	-	-	-	-
Diseases of G.U. and G.I. Tract	279	286	52	49	4	3	287	162	29	26	3	6	103	25	16	16	4	4
Diseases Bone and Joint	34	9	47	33	3	11	76	35	36	26	4	3	42	11	7	18	-	-
Allergic disorders	19	16	68	31	5	13	17	11	41	45	-	-	2	3	-	33	-	-
Thyroid disorders	51	27	51	44	2	7	42	17	26	41	2	-	7	5	14	-	14	20
Diabetes	12	22	42	50	-	-	54	48	33	15	7	8	32	18	16	6	3	11
Trauma	42	64	71	58	2	3	40	23	35	26	3	4	25	12	4	8	4	-
Hernia	13	3	77	33	8	-	34	4	32	25	3	-	9	3	11	33	-	-
Miscellaneous	446	246	41	39	5	5	388	194	31	29	5	4	212	82	11	6	1	6
Pregnancy	571	752	43	35	4	3	19	18	21	22	-	-	0	0	-	-	-	-
ALL DIAGNOSES	1700	1632	46	40	4	4	1286	800	31	27	5	5	691	311	13	11	3	5

TABLE 7. Shows the number of females and percentage of present and past cigarette smokers in each diagnosis, according to age and race.

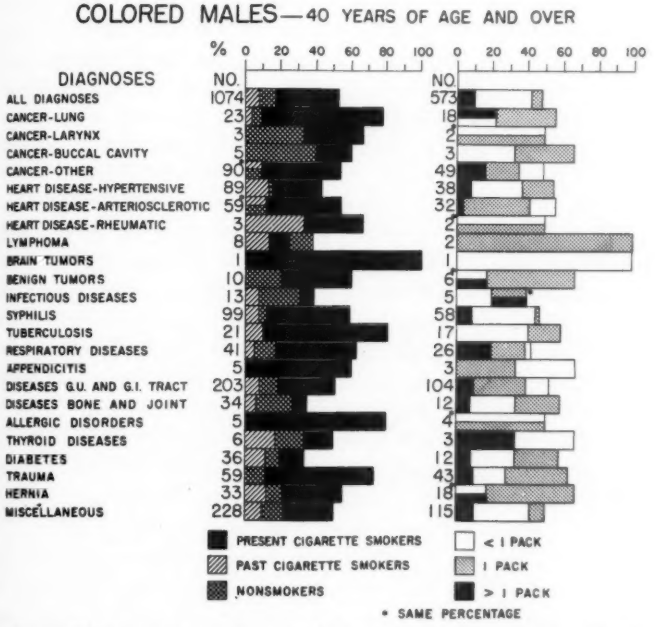


GRAPH I. Shows the number of white males 40 years of age and older in each diagnosis and in overlapping bars the percentage of present cigarette smokers, past cigarette smokers and nonsmokers; it also shows the number of present cigarette smokers and the percentage smoking less than one pack, one more than one pack daily.

and past cigarette smokers in the group of individuals with the different medical diagnoses. There were 62 white males 40 years of age and over with the diagnosis of lung cancer; of these 79 per cent were present cigarette smokers and 9.7 per cent were past cigarette smokers. Of these present cigarette smokers with lung cancer 10.2 per cent smoked less than a pack of cigarettes a day, 36.7 per cent smoked a pack, and 53.1 per cent smoked more than a pack. All of the less than a pack and the one pack smokers had smoked for 16 years or longer and all but 3.8 per cent of the more than a pack smokers had smoked for this length of time. Our data, like all other on this subject, show that patients with cancer of the lung are heavy cigarette smokers; however, 97.2 per cent of the white males in this study who had smoked cigarettes for

16 years or longer did not have lung cancer according to the medical record at the time of this survey. It would seem to us that little attention has been given in all of the surveys to those individuals that smoke and do not have cancer of the lung. Our findings support the opinion expressed by Schrek (1950)—"The relatively low percentage of deaths by cancer of the respiratory tract as compared to the high percentage of smokers indicates that smoking is, at most, only a weak carcinogenic agent."

Since we did not convert cigar and pipe smokers into cigarette smokers as Doll and Hill (1952) and Sadowsky *et al.* (1953) have done, our data will show a lower percentage of cigarette smokers and a smaller quantity of cigarettes smoked. To illustrate, a person who smoked 14 cigarettes and two cigars a day



GRAPH II. Shows the number of colored males 40 years of age and older in each diagnosis and in overlapping bars the percentage of present cigarette smokers, past cigarette smokers and nonsmokers; it also shows the number of present cigarette smokers and the percentage smoking less than one pack, one pack, and more than one pack daily.

was classified by us as a less-than-a-pack-a-day smoker; however, had we used Sadowsky's technique this same individual would have been classified as smoking 34 cigarettes a day, which would make the individual a heavy smoker.

We had 1,731 white males in our study who had smoked cigarettes for 16 years or longer; of these 21.3 per cent smoked less than a pack, 52.5 per cent a pack and 26.2 per cent more than a pack daily. Of these white males 2.8 per cent had cancer of the lung and 5.4 per cent hypertensive heart disease. It is difficult to explain, if smoking is a significant causal factor, why cancer of the lung is not more frequent in the female in this study in view of the fact that 34.0 per cent of 3,917 white females are cigarette smokers when compared to 67.8 per cent of 3,568 white males. Eight and six-tenths of these white females have smoked more than one pack of cigarettes as compared to 23.0 per cent of the white males. Furthermore, 34.9 per cent of the white females have smoked for 16 years or longer as compared with 58.2 per cent of white males. Cowdry (1955) in commenting on cancer of the lung in the male and female has this to say, "This difference is difficult to explain on the theory that females have been inveterate smokers for too short a time to develop lung cancer in view of a probably long period of delay in carcinogenesis. Many women now in their sixties have smoked cigarettes in large quantities for 15 to 20 years or more. The female death rate from bronchogenic carcinoma should rise as the male death rate rises if cigarette smoking is a major factor, but it does not." Dorn (1956) states that the mortality data for the United States in the year 1954 yield a ratio of male to female of 5.97 for the white population and 5.41 for the nonwhite population.

Of the 148 white male patients with hypertensive heart disease, 40 years of age and over, 61.5 per cent were present and 10.1 per cent were past cigarette smokers. Of the present cigarette smokers, 24.2 per cent had smoked less than a pack, 51.6 per cent a pack and 24.2 per cent more than a pack a day. Of the group of 81 white male patients with tuberculosis, 40 years of age and over, 80.2 per cent were present and 9.9 per cent were past cigarette smokers. Of the present cigarette smokers, 12.3 per cent had smoked less than a pack, 58.5 per cent one pack, and 29.2 per cent more than a pack daily. Obviously more indi-

viduals with tuberculosis smoked a pack of cigarettes or more a day than those with hypertensive heart disease.

There is one thing we consider very important in evaluating heart disease referable to smoking and that is to specify the type of heart disease to be included. Every physician recognizes congenital, bacterial, rheumatic, syphilitic and arteriosclerotic forms of heart disease. Also, cardiac hypertrophy, followed by myocardial failure, may accompany both pulmonary and systemic hypertension. Coronary arteriosclerosis, with or without thrombotic occlusion, may be included in the general classification of heart disease. If we are to relate cigarette smoking, as an etiologic factor, with one or all of these different types of cardiac disease, a hypothesis for such an association would be in order. Nicotine may cause a transient elevation in the blood pressure. Rats exposed to cigarette smoke, under conditions simulating human smoking, for their entire life-span showed no evidence either of a persistent hypertension or any cardiac lesions (Haag *et al.*, 1944). A persistent elevated blood pressure, such as we find in the so-called "benign hypertension," usually produces a hypertrophy of the left ventricle of the heart. This hypertrophy may be followed by dilation of the left ventricle and the patient is then considered to have myocardial failure—one type of heart disease. In coronary heart disease the primary process is one of arteriosclerosis. This may be accompanied by a thrombus that results in an occlusion with subsequent myocardial infarction. This is another form of heart disease. However, the mechanism of the action of cigarette smoking on this sclerotic coronary has not been satisfactorily presented as far as we know. McDevitt and Wright in 1955 stated that there is no evidence that tobacco is an etiologic factor in either peripheral arteriosclerosis, coronary artery disease, Raynaud's syndrome, cerebral vascular disease or hypertension. White and Sharber in 1934 analyzed the smoking habits of 750 patients with angina pectoris and concluded that neither the use of nor abstinence from tobacco played an important role in the genesis of angina pectoris.

Wynder in a book published in 1955 says, "The preliminary data of the American Cancer Society as reported by Hammond and Horn (1954) have suggested that cancer in general may be associated with tobacco smoking." This would imply either that there are multiple cancer-producing agents in cigarette tar or

that there is one agent that is totipotent. The term "cancer" as applied by Hammond and Horn would, in our opinion, include adenocarcinoma of the kidney, osteogenic sarcoma of the bone, malignant melanomas of the skin, adenocarcinoma of the prostate, squamous cell carcinoma of the male genitalia, and glioma, as well as cancer of the bronchi and lungs. In support of the theory that cigarette smoking causes cancer, Wynder (1955) cites the fact that "among 104 males with cancer of the esophagus, 96.0 per cent were smokers and 77.9 per cent smoked cigarettes either alone or with other forms of tobacco. The number of smokers is the same as found for lung cancer, but the use of cigarettes is less." Curwen, Kennaway and Kennaway (1954) show no increase in mortality from laryngeal cancer in England during the past 60 years although the consumption of cigarettes has markedly increased. In the United States the consumption of cigarettes is much higher than in England; however, the frequency rate of lung cancer is higher in England than it is in the United States (Russ, 1955).

From the available scientific data there is no carcinogenic agent in the group of approximately 400 known carcinogens (Cowdry, 1955; Hartwell, 1951) that can approach the multipotency of the hypothetical carcinogen implied to be present in cigarette tar. The frequency in which cigarettes are now smoked and have been smoked in this country and the fact that this problem of smoking and disease is not new but has been under investigation for more than 50 years would suggest the necessity for "scientific caution" in attempting to relate cigarette smoking as cause and effect to all cancers and to heart disease. Cowdry (1955) has said, "To have a common major factor in the direct production of two such different neoplastic and degenerative diseases would be surprising."

Our survey on smoking and disease, as well as all other similar studies, indicates the necessity of establishing categories for different types of smokers, the amount they smoke, and the length of time they have smoked. When there occurs a variation in the relative risk of developing lung cancer of 1.9 to 79.0 for heavy smokers in a group of only 14 studies (Cutler, 1955), it is obvious that a closer agreement in such data would be desirable in any attempt to establish the relation of smoking to disease.

Summary

The smoking data on 12,050 individuals are correlated with their medical diagnoses. A very high percentage of the white males with cancer of the lung are heavy cigarette smokers; however, a number of the other disease categories show about the same percentage as does lung cancer. Ninety-seven and two-tenths per cent of the white males in this study who had smoked cigarettes for 16 years or longer did not have lung cancer according to the medical record at the time of this survey.

The data summarized in Graphs I and II show the frequency of present and past cigarette smokers and nonsmokers and the amount of cigarettes smoked by both the white and colored males 40 years of age and over. The white male who has cancer of the lung is a much heavier smoker than the colored male who has this same neoplasm. Approximately the same percentage of white males with benign neoplasms and with cancer of the lung smoke more than one pack of cigarettes a day. The smoking habits of the white males with diabetes are essentially the same as those with cancer. The majority of white males in this survey smoke a pack of cigarettes or more a day.

Since such a high percentage of adults smoke any correlation between smoking per se and a specific disease should be carefully evaluated. The wide variation in the results of the 14 studies referable to the relative risk of developing lung cancer by smokers (1.9 to 79.0 for heavy smokers) would suggest to us that some of it may be due to differences in techniques. It would seem to us that additional data are needed to establish the percentage of individuals that smoke and do not have cancer of the lung or heart disease. The present study was made to help evaluate this phase of the smoking controversy.

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[a] Laboratory of Experimental Pathology, The University of Texas Medical Branch, Galveston, Texas. This is a cooperative study with the Medical College of Virginia. We wish to acknowledge the assistance and cooperation received from the hospital administrations and their respective staffs of the Veterans Administration Hospital, Houston, Texas, the U. S. Public Health Service Hospital and John Sealy Hospital, Galveston, Texas,, and from the IBM Department of The University of Texas Medical Branch. Accepted November 10, 1957.

EFFECT OF BEHAVIOR-ALTERING DRUGS ON ALCOHOL CONSUMPTION BY RATS[a]

LORENE L. ROGERS AND RICHARD B. PELTON

For many years we have been interested in the problem of alcohol consumption and the basic factors which underlie an excessive appetite for alcohol. We have shown (1) that rats on marginal diets consume more alcohol than those on diets abundantly supplied with all the known vitamins. It has also been demonstrated that glutamine, an amide of an amino acid, not only overcomes the toxicity of ethyl alcohol for certain microorganisms (2) but, when added to the stock diet of laboratory rats, will lower the voluntary alcohol consumption of these animals (3, 4). Studies with human beings indicate that both vitamin supplementation (5) and glutamine administration (6) show promise in preventing compulsive drinking in many alcoholics.

Since the recent upsurge of interest in the so-called behavior-altering drugs, a number of workers have reported some success in controlling compulsive drinking in human alcoholics by administering one or another of the tranquilizing drugs (7). By determining the effect of these drugs as well as that of certain central nervous system stimulants on alcohol consumption by rats, we hoped to gain further insight into the causes of addiction to alcohol.

Experimental

Experimental design was similar to that described in earlier studies (1, 3), rats of Wistar strain approximately 6 months of age being used. Each rat was caged separately and given a choice of two drinking bottles, one containing water and the other 10 per cent alcohol. The bottles were checked, emptied and refilled, and the positions of the bottles changed every three to five days. The average daily alcohol consumption for each rat was determined during a three-week period while all the animals were maintained on ground Purina lab chow. The animals were then divided into groups of ten each in such a way that the average

alcohol consumption for each group was approximately equivalent. To the diets of seven of these groups were added the tranquilizing drugs Thorazine (chlorpromazine), Sparine (promazine), Reserpine, Frenquel (azacyclonol), and Miltown (meprobamate) and the central nervous system stimulants Meratran (pipradrol) and Ritalin (methylphenidylacetate HCl) respectively (7) and the alcohol consumption of each rat was checked for another three-week period. Dosage was determined according to the best data then available on human dosage, approximately ⅓ the human dosage being used. An eighth group was kept on the stock diet to serve as controls. At the end of the three-week period all experimental animals were returned to the stock diet and alcohol consumption was checked for an additional four weeks. In a separate experiment, the effect of lysergic acid diethylamide was similarly determined. Results are shown in table 1.

Discussion

Statistical analysis (Student's T test) shows no significant effect on alcohol consumption from any of these drugs during

TABLE 1

Effect of Behavior Altering Drugs on Alcohol Consumption by Rats

No. Rats Used	Substance Added	Alcohol Consumption (ml. 10 per cent alcohol/100g. rat/day) Control Period 3-Weeks	Experimental Period 3-Weeks	Per Cent Change From Control Period	Post-Experimental Period 4-Weeks	Per Cent Change From Control Period
10	Reserpine 0.3 mg./day	2.74	2.41	—12	2.27	—17
10	Thorazine 15 mg./day	2.68	3.29	+23	3.40	+26
10	Sparine 15 mg./day	2.75	5.61	+104	3.48	+27
10	Frenquel 6 mg./day	2.68	3.13	+16	2.61	— 3
10	Miltown 480 mg./day	2.77	3.40	+23	1.56	—44
10	Meratran 1.5 mg./day	2.72	2.54	— 6	2.84	+ 4
10	Ritalin 25 mg./day	2.75	3.54	+29	2.66	— 3
10	None	2.70	2.79	+ 3	2.82	+ 4
		3-Weeks	5-Weeks	Per Cent Change	3-Weeks	Per Cent Change
10	LSD 10μg/day	3.45	3.08	—11	3.10	—10
10	None	3.53	3.94	+12	4.02	+14

either the experimental period or the post-experimental period except in the case of Sparine. No explanation can be given at the moment for the increased consumption in the Sparine group. The compound was apparently distasteful to the animals, for they kicked out much of their food, and it might be supposed that they were replacing dietary calories with alcohol. However, the group on Miltown kicked out even more of their food without a corresponding increase in alcohol consumption. In fact, the apparent increase in consumption in the group on Miltown in the experimental period and corresponding decrease in the post-experimental period is due more to weight changes than to change in absolute amounts of alcohol consumed. This same pattern is not seen in the group on Sparine.

Except for the anomalous effect of Sparine, results with the other compounds seem to indicate that neither sedation nor stimulation of the central nervous system has a significant effect on alcohol consumption by rats maintained throughout on a stock diet of Purina lab chow—at least at the dosage levels used here. Inasmuch as either vitamin or glutamine supplementation decreases alcohol consumption in rats and both treatments are also effective in preventing compulsive drinking in many human alcoholics, we may reasonably conclude that they are in some way exerting an effect on the metabolic pathways involved in controlling appetite for alcohol. Since the tranquilizing drugs, on the other hand, show no effect in rats but appear to in human alcoholics, it may be assumed that these drugs have no direct effect on these metabolic pathways but are merely exerting a quieting influence which substitutes for the sedation obtained by consuming alcohol. While such drugs may be effective in the treatment of alcoholics during the withdrawal period or in tiding them over periods of emotional tension, it would seem that a truly effective and permanent control must do more than merely exert a calming effect.

Summary

Five tranquilizing drugs were ineffective at the dosage levels used in reducing alcohol consumption by rats on a regular stock diet. One of the drugs caused a significant increase in alcohol consumption. Neither of two central nervous system stimulants

nor lysergic acid diethylamide significantly altered alcohol consumption.

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7. No attempt is made to list all the papers in this field. See Abstracts of Current Literature in recent issues of Quarterly Journal of Studies on Alcohol.
8. Reserpine and Ritalin were generously supplied by Ciba Pharmaceutical Products, Inc.; Thorazine by Smith, Kline & French Laboratories;

Sparine by Wyeth Institute for Medical Research; Frenquel and Meratran by the Wm. S. Merrell Company; and Miltown by Wallace Laboratories, and to each of them we express our grateful appreciation. Lysergic acid diethylamide was purchased from Sandoz Pharmaceuticals.

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STUDIES ON ASCORBIC ACID IN BLOOD. I. EXCHANGE OF ASCORBIC ACID BETWEEN ERYTHROCYTES AND PLASMA[a]

LOTHAR L. SALOMON[b]

In studying the transfer of ions across membranes in biological systems, problems occasionally arise which are difficult to resolve, particularly with adherence to physiological conditions, unless isotopically-labeled materials are used. Thus, *net* transfer may be the only sign that migration of a substance has occurred at all, and this gives information restricted to differences between inflow and outflow. Migration in the complete absence of changes in concentration of the migrating material can be readily detected with isotopically-labeled materials, and suitable arrangement of experimental conditions may permit interpretation of such experiments in terms of *total* flow. The availability of ascorbic acid-1-C^{14} offered an opportunity to reinvestigate certain aspects of the exchange of ascorbic acid between plasma and erythrocytes which were difficult or impossible to examine before. It was hoped to place on a sounder basis certain aspects of the dynamics of ascorbic acid in blood which had been studied mostly *in vitro* and on which complete agreement did not exist.

The possibility has been considered that erythrocytes may be nearly, if not entirely, impermeable to ascorbic acid (2). However, that ascorbic acid penetrates the membranes of blood cells of scurvy-liable species is apparent from the fact that the cells lose ascorbic acid in deprivation, and store ascorbic acid in realimentation. Studies such as those by Butler and Cushman established that changing concentrations of ascorbic acid in plasma are more rapidly reflected by parallel changes in erythrocytes than leucocytes (3). Although scurvy-resistant species have received little attention, the present and other results (27) show that the red cells of such animals are similarly permeable to ascorbic acid.

In humans, administration of test doses of ascorbic acid result in increased concentrations in erythrocytes (8, 10). *In vitro*, preparations of erythrocytes did not exhibit changes in concen-

tration over a fairly wide range in concentration of ascorbic acid in the suspending medium (8). Heinemann (12) and Lloyd (19), however, suggested that when leucocytes are also present, demonstrable changes in cellular concentration of ascorbic acid do occur *in vitro* as well. Beyond that, they differed widely in their conclusions, for Heinemann held that leucocytes exhibit uptake of ascorbic acid, contrary to an earlier paper, in which the changes in concentration were assigned to erythrocytes (10), while Lloyd indicated that erythrocytes, but not leucocytes, exhibit net uptake. Lloyd further suggests that leucocytes oxidaze ascorbic acid. In the rat, leucocytes accumulate radioactivity only slowly after administration of radioactive ascorbic acid (27). If the presence of leucocytes is required for uptake of ascorbic acid in human blood cells, the rôle of leucocytes must be different in this respect in the rat. But Kellie and Zilva found human leucocytes also to be indifferent toward ascorbic acid, neither catalyzing oxidation nor reduction (17).

The possible occurrence of dehydroascorbic acid in blood is of significance in connection with the present study. The instability of ascorbic acid, and the difficulty of reliably measuring small concentrations of dehydroascorbic acid, indicate that dehydroascorbic acid may occur as an artifact rather than a naturally-occurring metabolite (2, 4). The conversion of ascorbic acid to dehydroascorbic acid in blood has been claimed by Lloyd (19) and Panteleva (23), but to products other than dehydroascorbic acid by Heinemann (12). Entry into white cells would probably be a prerequisite for such transformations but, as pointed out, radioactive ascorbic acid did not enter leucocytes very readily (27). Although some have noted significant concentrations of dehydroascorbic acid in blood (5, 23), other results demonstrate almost complete or total absence of the reversibly-oxidized form of ascorbic acid (2, 4, 18, 6).

Most animal tissues are capable of reducing dehydroascorbic acid (17, 1), a fact also clearly shown by the excretion of reduced ascorbic acid following administration of dehydroascorbic acid (2, 15). Such a reducing system may also exist in blood (23, 26), but Barron *et al.* (1) and Borsook *et al.* (2) doubt this. Nor does permeation of dehydroascorbic acid into blood cells (19, 23) constitute proof of the importance of such a process *in vivo*.

General agreement exists that erythrocytes stabilize ascorbic

acid (2, 11, 12, 17, 19, 23), but not on the mechanism by which stabilization is accomplished. Borsook *et al.* presented evidence that the responsible system functions by preventing the oxidation of ascorbic acid, rather than by salvaging of dehydroascorbic acid (2). Ascorbic acid is also more stable in whole blood than in plasma or serum (7, 16, 24).

Information from this study indicates that ascorbic acid, administered as ascorbic-1-C^{14} acid, entered erythrocytes at a rate which was rapid in comparison with the normal level of ascorbic acid in erythrocytes, but did not involve net uptake. The assumption that reduced ascorbic acid traversed the membrane of erythrocytes in this case (which is not meant to imply that dehydroascorbic acid cannot do the same) leads to an interpretation of the data which is consistent with all the present, as well as the preponderance of previously-recorded results. The rate of this exchange was determined. An apparent discontinuity or interference in exchange seems to be caused by the existence of two forms of ascorbic acid in erythrocytes, differing in the rate at which they accumulate radioactivity from ascorbic acid in plasma.

Experimental

Male Sprague-Dawley rats, weighing from 125 to 157 gm., were maintained on Purina laboratory chow and water, *ad libitum*, until sacrifice. These animals were hypophysectomized since simultaneous studies on the adrenal glands of these rats were performed. Experiments were carried out 5 to 6 days postoperatively. At the time intervals prior to decapitation indicated in figure I and and table 2, an aqueous solution of ascorbic-1-C^{14} acid (28) was administered intraperitoneally. The material had a specific activity of 2.35×10^6 counts per min. per mg. (7.04×10^6 counts per min. per mg. absolute, based on standards prepared from National Bureau of Standards Beta Ray Standards). Counting procedures were as reported previously (32). The administered doses were close to 1 mg. In order to make the final data comparable, counting data were normalized to 1.00 mg. of injected radioactive ascorbic acid after correction for background and self-absorption. This involved multiplication of the counting rate (after correction for background) by

$$\frac{1}{\text{mg. of radioactive ascorbic acid administered}}.$$

Immediately upon collection of blood in beakers containing heparin, the samples were centrifuged to minimize further exchange of ascorbic acid between plasma and cells. The plasma was then separated, the layer of white cells removed, and erythrocytes withdrawn from well below the surface.

Samples for determination of ascorbic acid were prepared by adding 3 volumes of 8 per cent trichloroacetic acid to erythrocytes, and 5 per cent

trichloroacetic acid to plasma. The clear supernate obtained after centrifugation was treated by the method of Lowry *et al.* (20) for the measurement of total ascorbic acid, except that the proportion of samples and reagents was so chosen as to give a final volume of 2.7 ml. In testing this method, it was found that samples from extracts of erythrocytes developed turbidity during incubation, which was not removed by addition of sulfuric acid, and consequently was not osazone. Plasma gave no similar problem. Since changes in concentrations of reagents used at various stages of the procedure did not result in improvement, the suspended material was removed routinely by filtration of such samples through fine-porosity sintered glass funnels. Sufficient time after addition of sulfuric acid for all osazone to become solubilized was allowed. Under these circumstances, standard samples gave unchanged optical density readings over the wide range of concentrations tested, showing that interfering substances only were removed.

In calculating the concentration of ascorbic acid, allowance was made for the residue precipitated by trichloroacetic acid, by subtracting from the total volume the weight of residue. The weight was determined after drying at 100° C. for 24 hours, and cooling over $CaCl_2$ *in vacuo*. An additional correction for the presence of plasma in the specimens of erythrocytes was made, as shown below.

The specific activity of ascorbic acid in erythrocytes can be obtained by dividing counts per min. per unit amount of erythrocytes (or extract) by the ascorbic acid contained in unit amount of the same sample. The same procedure can be used to determine specific activity of ascorbic acid in plasma. This is possible, because it has been shown previously that whole blood (30), and systemic plasma (31) contains only radioactivity as ascorbic acid under the conditions of these experiments. Additional data, showing that this applies to erythrocytes of rats as well, are presented in table 1. However, these

TABLE 1

Identity of Radioactive Material in Erythrocytes of Rats Following Injection of Ascorbic-1-C^{14} Acid

Expt.*	Time After Injection	Radioactivity in Total Extract	Radioactivity in Ascorbic Acid†	Radioactivity Isolated as Ascorbic Acid
		Counts Per Min.	Counts Per Min.	Per Cent
1	30 min.	3,600	3,300	91.7
2	12 hrs.	13,600	13,500	99.2

* Each sample consisted of blood pooled from three rats.
† These values represent the specific activity of isolated ascorbic acid, multiplied by the tatal amount of carrier originally present.
The radioactivity found in extract of experiment 1 was low, so that a counting error of ± 10 per cent must be taken into consideration.

samples of erythrocytes contained approximately 3 per cent of intercellular plasma (21, 36). The similarity of concentrations of ascorbic acid in erythrocytes and plasma indicates that the "actual" and the "measured" concentrations of ascorbic acid in erythrocytes should not be appreciably different due to error from this source. Since *specific activities* in plasma and erythrocytes

may differ widely, however, it was necessary to abolish error from this source. The following equation was used in correcting for the presence of 3 per cent intercellular plasma in erythrocyte samples[c]:

$$(1) \qquad r_c = \frac{R_s - 0.03\,R_p}{A_s - 0.03\,A_p}$$

The corrected "actual" concentration of ascorbic acid in erythrocytes was calculated by means of the following equation:

$$(2) \qquad A_c = \frac{100}{97}(A_s - 0.03\,A_p)$$

Results and Discussion

In addition to investigating the nature of radioactive material found in whole blood and systemic plasma (30, 31), radioactivity in erythrocytes was also examined. As shown in table 1, erythrocytes contain virtually no radioactive substances other than ascorbic acid. The isolation of ascorbic acid was by a previously reported procedure (32), except that H_2S was allowed to pass through the trichloroacetic acid solution while the extraction was in progress.

As shown in table 2, the concentration of ascorbic acid in plasma remained high throughout the period covered by these data. Subsequently, however, the concentration declined and approached a "normal" level (32). This indicates that ascorbic acid rapidly moved into plasma following intraperitoneal injection. It is not surprising, under these circumstances, that radioactivity in plasma also accumulated rapidly. In fact, maximal specific activity of ascorbic acid in plasma was attained within five minutes after administration of radioactive compound. Initially, therefore, changes in concentration were paralleled by changes in specific activity. During the ensuing 45 minutes, the concentration remained fairly constant, whereas the specific activity decreased markedly. This suggests an exchange between ascorbic acid in tissues and plasma, without net transfer into tissues. Data for erythrocytes provide more concrete evidence that such a process may, indeed, occur.

The concentration of ascorbic acid in erythrocytes remained substantially unaltered throughout these experiments (*see* Table

TABLE 2

Ascorbic Acid in Erythrocytes and Plasma Following Administration of 1 mg. of Ascorbic-1-C^{14} Acid

	Time after injection of ascorbic acid, in min.								
	5	10	15	20	25	30	35	40	50
	Ascorbic acid in erythrocytes, in mg. per 100 gm.*								
	.59	.72	.68	.77	.70†	.73	.73	.67†‡	.63
	.56	.54	.60	.65	.62†‡	.65	.71		.53
	.67	.56	.66	.57		.73	.68		.66
	.70	.61	.51	.64		.60	.57		.63
	.60	.61	.50	.67			.54		.64
			.58†‡	.51			.53‡		.71
			.42‡				.57‡		.67
			.61‡				.52‡		.67
									.67†‡
Average (weighted)	.62	.61	.57	.63	.66	.68	.61	.67	.65
	Ascorbic acid in plasma, in mg. per 100 gm.§								
	1.52	1.55	1.25	1.08	1.25†	1.02	2.00	1.32†‡	1.14
	1.23	1.01	1.07	1.29	1.55‡†	1.48	1.68		.78
	2.38	1.83	1.37	1.97		2.11	1.64		.94
	1.75	1.56	1.51	1.54		1.46	1.47		1.48
	2.15	1.71	1.67	1.44			1.45		1.10
	1.69	1.81	1.49	2.01			1.34		.92
			1.33†‡				2.12		2.29
			1.35				1.13‡		2.15
			1.60				.94‡		1.48
							1.34‡		1.38
									.91
									1.76
									1.30†‡
Average (weighted)	1.79	1.58	1.39	1.56	1.38	1.52	1.51	1.32	1.35

* Corrected for intercellular plasma.
† Pooled sample, representing specimens from three rats.
‡ Intact rats; all others hypophysectomized.
§ Additional data, relating to longer periods after administration of ascorbic acid, have been previously published (32). By comparison of these with the present results, the transitory, but marked increase in concentration of ascorbic acid in plasma becomes more evident.

2). The fact that it remained unaffected by changing concentration of ascorbic acid in plasma indicates the operation of processes tending to maintain the concentration of intracellular ascorbic acid at a constant value. In experiments *in vitro*, changes in concentration of ascorbic acid in the suspending medium over a considerable range, 0 to 4.60 mg. per 100 ml., did not result in detectable movement across the membrane of human erythrocytes, although such movement appeared to take place *in vivo* (8). Erythrocytes in rats may differ from human erythrocytes in that respect.

While net changes in concentration of ascorbic acid in erythrocytes were not detectable, exchange with ascorbic acid in plasma occurred at an appreciable rate. The data of table 3 and figure I show that ascorbic acid was transferred into the cells, since radioactivity accumulated rapidly. However, the flow of ascorbic acid into the cells must have been balanced by an equal flow outward. Exchange of ascorbic acid appears to be independent of its concentration in plasma. This is analogous to exchange of K^+, which traversed the membrane, but at a rate which was not influenced by its concentration in plasma over the range studied (25, 35, 36).

Curve B, figure I, shows the change in specific activity of ascorbic acid in erythrocytes following administration of radioactive ascorbic acid. The remarkable aspect of this curve lies in the fact that it indicates an initially rapid increase in specific activity, but an increase which fails to be sustained. The curve soon ceases to rise, before it has intersected the curve for the specific activity of ascorbic acid in plasma (curve A, Fig. I). Were the exchange to proceed unhampered, it is to be anticipated that the specific activity of ascorbic acid in erythrocytes should increase until it equals that of plasma. The observations indicate that some interfering factor exists.

Effect of Neogenesis of Ascorbic Acid. The precursor of ascorbic acid in rats is probably glucuronic acid (9, 14). If ascorbic acid were synthesized in erythrocytes, the specific activity of radioactive ascorbic acid would be constantly diluted by the addition of non-radioactive ascorbic acid, the degree of dilution being dependent both upon the rate at which ascorbic acid was synthesized from (non-radioactive) glucuronic acid, as well as the rate at which cellular ascorbic acid exchanged with extracellular ascorbic acid. Although a potential source of error in related cases, it has been possible to show that this factor can be eliminated in the present instance; other rate measurements have shown that ascorbic acid may probably be synthesized by erythrocytes, but at a rate so slow that it becomes entirely negligible in comparison with the rate of exchange (27).

Dehydroascorbic Acid as the Exchanging Substance. It appears well established that erythrocytes do not contain concentrations of dehydroascorbic acid large enough to account for the observed amount of radioactivity. Thus, *if* dehydroascorbic acid enters

preferentially, it must be quickly reduced. Hence, it is not probable that dehydroascorbic acid can exist as a separate radioactive pool in these cells, along with a non-radioactive pool of ascorbic acid.

However, if the assumption is made that ascorbic acid is oxidized in plasma, and that the product of oxidation traverses the membrane of erythrocytes, to be reduced during or immediately after entry into the cells, one must arrive at substantially the same conclusions as those discussed in the next section. This newly-added ascorbic acid apparently does not "mix" with the major fraction of cellular ascorbic acid, and so indicates the existence of two kinds of ascorbic acid in erythrocytes.

It will be assumed that it is ascorbic acid which exchanges, without net change in concentration of cellular ascorbic acid. This allows interpretations to be made which are in agreement with much of previously-recorded work, and which appear to be less equivocal in other regards. It is recognized, however, that the identity of the exchanging substance cannot be definitely settled without further studies; ascorbic acid has been implicated here chiefly on the basis of circumstantial evidence.

Effect of Incomplete Exchange. Incomplete exchange may arise either by discontinuous exchange, or by the unavailability for exchange of a fraction of ascorbic acid in erythrocytes. There is nothing in these data, nor in other related studies, to suggest that exchange was discontinuous. However, existence of bound ascorbic acid in blood cells (13) and in human erythrocytes (33) but not in serum (12) or plasma (34) has been reported.[d] Since it is reasonable to assume that bound ascorbic acid would exchange at a slower rate than free ascorbic acid, an explanation for the present findings can be made on that basis.

A situation may be considered in which a rapidly—and a slowly—exchanging form of ascorbic acid exist in erythrocytes. The rapidly-exchanging fraction of ascorbic acid quickly approaches the specific activity of ascorbic acid in plasma. The other, slowly-exchanging fraction, does not accumulate an appreciable amount of radioactivity in the initial stages after injection of radioactive compound. The measured specific activity[e] would then be lower than the specific activity of the rapidly-exchanging fraction alone, in direct proportion to the ratio of radioactive ascorbic acid to total ascorbic acid in the cells.

The occurrence of a maximum in the specific activity of ascorbic acid in erythrocytes would not be possible unless some fraction had reached the specific activity of ascorbic acid in plasma at that point. This is simply a consequence of the fact that continuous exchange requires the specific activity of cellular ascorbic acid to increase until it equals the specific activity of ascorbic acid in plasma (provided neogenesis does not interfere), and to decrease thereafter, if specific activity of ascorbic acid in plasma decreases. The specific activities, as here measured, may be considered to be average values for radioactive rapidly-exchanging, and non-radioactive slowly-exchanging ascorbic acid. The "true" specific activity of rapidly-exchanging ascorbic acid can be estimated as shown below; it must be higher than the observed, average value in direct proportion to the ratio of concentrations of total and rapidly-exchanging ascorbic acid.

Representing the measured specific activity by

$$r_{c,av.} = \frac{R_c}{A_{c,f} + A_{c,b}}$$

and since all radioactivity is considered to reside in the rapidly-exchanging fraction,

$$r_{c,f} = \frac{R_c}{A_{c,f}}$$

Combining the two expressions, one obtains

$$(3)\ r_{c,f} = \frac{r_{c,av.}\ (A_{c,f} + A_{d,b})}{A_{c,f}}$$

Determination of the ratio of free to bound ascorbic acid is most simply made at the maximum of the specific activity of ascorbic acid in erythrocytes. At that point, the specific activity of the rapidly-exchanging form must equal that of ascorbic acid in plasma. This occurred approximately 16 minutes after injection of radioactive ascorbic acid[f], at which point the observed (average) specific activity for the cells was 155×10^3 counts per minute per mg. of ascorbic acid, while the true specific activity was 615×10^3 counts per minute per mg. of ascorbic acid (equal to the specific acivity of ascorbic acid in plasma). Hence the ratio of free to total ascorbic acid is $155 \times 10^3 / 615 \times 10^3 = 0.25$.

The average concentration of ascorbic acid in erythrocytes in this and related studies was found to be 0.60 mg. per 100 gm. of cells (S.D. 0.08 mg. per 100 gm., 102 determinations), and the concentration of free ascorbic acid, based on that value, 0.15 mg. per 100 gm.

With equation *(3)* it now is possible to evaluate all true specific activities up to the point when slowly-exchanging ascorbic acid acquired an appreciable amount of radioactivity. The upswing of curve B, figure I, (probably due to appreciable uptake

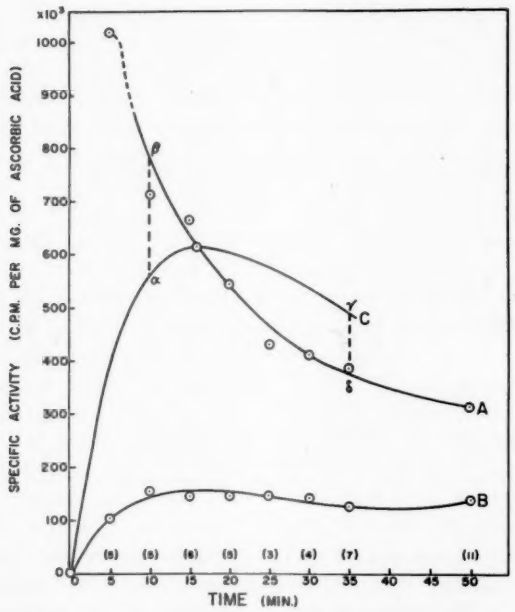

FIG. I. Change with time of radioactivity in ascorbic acid of plasma and erythrocytes of hypophysectomized rats, following administration of radioactive ascorbic acid. Dose: 2.35×10^6 counts per min. (7.04×10^6 counts per min., absolute; 1.00 mg). Curve A represents the average for ascorbic acid in plasma of all rats. Curve B represents observed values for cellular ascorbic acid. Curve C represents the "true" specific activity values for cellular rapidly-exchanging ascorbic acid. All specific activities corrected for background and self-absorption, but are not absolute. The upswing at the 50 min. period, which will continue for several hours, is a sign of the by now significant accumulation of radioactivity in the slowly-exchanging fraction of ascorbic acid of erythrocytes. Numbers in parentheses indicate number of rats used in experiment.

of radioactivity by the slowly-exchanging fraction), indicates that true specific activities cannot be calculated with reliability beyond approximately 35 minutes after injection of radioactive ascorbic acid. Curve C, figure I, which represents the calculated true specific activities of rapidly-exchanging ascorbic acid in erythrocytes, is discontinuous for that reason.

Calculation of the Rate of Exchange. The rate of exchange of ascorbic acid between erythrocytes and plasma can be calculated from the available data. The following assumptions, most of them discussed above, will be made:

1. Ascorbic acid is not being synthesized in the cells in significant amounts.
2. The concentration of ascorbic acid remains constant in erythrocytes.
3. The rate of exchange is independent of ascorbic acid in plasma.
4. All radioactivity in plasma and erythrocytes is present as ascorbic acid.
5. Entering radioactivity mixes uniformly with all rapidly-exchanging ascorbic acid.
6. Slowly-exchanging ascorbic acid possesses a negligible amount of radioactivity during the early phases.

Under these circumstances, the time-rate of change of the concentration of cellular ascorbic acid is

$$\frac{dA_c}{dt} = K - K' = O$$

where K represents the constant rate at which ascorbic acid enters the cells, and K' the sum of the constant rates at which ascorbic acid leaves (unchanged, or in altered forms, or in both ways). Since total quantity $\times$ specific activity = total radioactivity, the radioactivity entering and leaving the cells is

$$K_1 (r_p - r_c) = A_c \frac{dr_c}{dt} \quad \text{where } K_1 = K = K'.$$

K_1 can now be estimated by evaluation of the integral of the expression

$$(4)\ \frac{A_c \Delta r_c}{K_1} = \int r_p\, dt - \int r_c\, dt.$$

The integrals at the right represent the area (α, β, δ, δ) of figure I. The limits of integration were chosen to be the 35-minute and 10-minute periods, since the erythrocyte curve becomes unreliable beyond the former period, and the plasma curve unreliable before the latter.

Based on this calculation, exchange takes place at the rate of 1.1×10^{-2} mg. per min. per 100 gm. of erythrocytes. This represents an appreciable fraction of the rapidly-exchangeable form (7.3 per cent), as well as of the total amount of ascorbic acid in erythrocytes (1.8 per cent). It indicates that the turnover time of rapidly-exchangeable ascorbic acid is less than 14 minutes. The turnover time is, however, of limited significance in this case, since the major portion of cellular ascorbic acid turns over at a much slower rate (27).

It may be mentioned that the existence of two forms of ascorbic acid in erythrocytes requires the existence of a bimodal specific activity-time curve for cellular ascorbic acid. One of the two maxima, representing that for rapidly-exchanging ascorbic acid, has been discussed above. The maximum for slowly-exchanging ascorbic acid has now also been located (27) providing additional support for the interpretations.

This rapid rate of exchange indicates that ascorbic acid enters and leaves erythrocytes facilely. The mechanism by which this process occurs, and by which interacellular concentration of ascorbic acid is maintained constant, cannot be deduced from these data alone.

So as to ascertain the effect of hypophysectomy on exchange of ascorbic acid between plasma and erythrocytes, similar experiments were performed on normal rats, descendants of the Sprague-Dawley strain, obtained from the local colony. Specific activities determined at 15, 25, 35, 40, and 50 minutes, respectively, after injection of ascorbic acid, were virtual replicates of specific activities determined on samples obtained from hypophysectomized rats. These data, shown in table III, demonstrate that hypophysectomy did not affect the rate of exchange of ascorbic acid between plasma and erythrocytes, and that the conclusions here reached apply to normal, as well as hypophysectomized rats.

TABLE 3

Specific Activity of Ascorbic Acid in Erythrocytes and Plasma of Normal Rats, Following Administration of 1 mg. of Ascorbic-1-C^{14} Acid

	Time after injection of ascorbic acid, in min. 15	25	35	40	50
	Counts per min. per mg. of cellular ascorbic acid, in thousands*				
	129†	129†	144	125†	113†
	138		146		
	173		149		
Average (weighted)	140	129	146	125	113
	Counts per min. per mg. of plasma ascorbic acid, in thousands				
	600†	583†	392	456†	293†
	513		272		
	623		362		
Average (weighted)	587	583	342	456	293

* Corrected for intercellular plasma.
† Pooled sample, respresenting specimens from three rats.

Summary

Ascorbic acid has been found to enter erythrocytes of rats at a rapid rate. In the absence of significant changes in concentration of cellular ascorbic acid paralleling those in plasma, it was concluded that no net transfer was involved.

It was concluded that two forms of ascorbic acid exist in erythrocytes of rats, one accumulating radio-activity at a rapid rate from radioactive ascorbic acid in plasma, the other accumulating radioactivity at a much slower rate. The rapidly-exchanging form constitutes about 25 per cent of ascorbic acid in erythrocytes.

The rate at which ascorbic acid exchanges between plasma and the rapidly-exchanging form in erythrocytes was calculated to be 1.1×10^{-2} mg. of ascorbic acid per 100 gm. of cells. Hypophysectomy does not affect this rate.

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[c] The following symbols will be used:
r = specific activity of ascorbic acid, in counts per min. per mg.
A = concentration of ascorbic acid, in mg. per 100 gm. of material.
R = radioactivity, in counts per min. per 100 gm. of material.
The subscripts:
c = cell
p = plasma
s = sample (e.g., sample of erythrocytes plus intercellular plasma).
c, av. = average value for cells
c, true = true value for cells
c, f refers to cellular rapidly-exchanging (free) ascorbic acid
c, b refers to cellular slowly-exchanging (bound) ascorbic acid

[d] Matusis (22) has reported failure to find bound ascorbic acid in the supernate when blood was treated with trichloroacetic acid or metaphosphoric acid.

[e] It may be noted that the methods of assay used do not distinguish between the two forms of ascorbic acid, and simply measure radioactivity and ascorbic acid per unit quantity of cells.

[f] The location of the maximum of curve B, figure I, is not at once apparent from the data. However, if the maximum is properly designated, the value of K, should be constant wherever along curve C, figure I, it is measured. This condition is fulfilled by locating the maximum as shown.

STUDIES ON ADRENAL ASCORBIC ACID. III. EXCHANGEABILITY WITH EXTRACELLULAR ASCORBIC ACID[a]

LOTHAR L. SALOMON[b]

Partial results of a program of investigation aimed at elucidating certain aspects of the enigmatic position of ascorbic acid in metabolism, and especially in the adrenal glands, have been previously published (3, 4, 5). This paper represents a summary of some additional data on the same general problem.

Systematic measurements on the exchangeability of ascorbic acid in adrenal glands were undertaken. This was prompted, in part, by the finding that radioactive ascorbic acid appeared to be affected in ways different from total adrenal ascorbic acid under well-defined experimental conditions. Thus, the amount of radioactivity in adrenal glands of rats which had received radioactive ascorbic acid remained unaffected by injection of ACTH (adrenocorticotrophic hormone), although the concentration of ascorbic acid decreased considerably. As a result of these investigations, it could be shown that two "forms" of ascorbic acid exist within adrenal glands, differing sharply in the rate with which they acquire radioactivity originating from ascorbic acid in plasma.

Because of the relatively slow rate of exchange of one of these forms, it is suggested that it represents (protein-) bound ascorbic acid, whereas the other, rapidly-exchanging form may represent free ascorbic acid. It is of considerable interest that the free form remains indifferent to the action of ACTH, and that the hormone-induced depletion of adrenal ascorbic acid appears to take place solely at the expense of the bound form.

Additional evidence now supports the contention (4) that changes in permeability or other non-specific effects cannot explain the action of the hormone on adrenal ascorbic acid.

METHODS AND MATERIALS

Measurement of specific activities of ascorbic acid in adrenal glands and plasma, and other procedural details have been outlined previously (4, 5). Male, hypophysectomized rats of the Sprague-Dawley strain, ranging in weight from 125–157 gm., were used 5–6 days postoperatively. These animals received Purina laboratory chow and water, *ad libitum*.

Ascorbic-1-C^{14} acid (6) was administered intraperitoneally in aqueous solution, in quantities close to 1 mg. (7.04×10^6 counts per min. per mg., absolute). To permit comparison of the data, all specific activities were normalized to a dose of 1.00 mg., as shown before (5, 7). ACTH (Armour) was administered intraperitoneally in aqueous solution, in doses of 3 I. U. per rat. Control animals received no ACTH.

The procedure consisted of injection of ACTH to the test groups, followed by ascorbic-1-C^{14} acid after a lapse of 45 minutes. At that time, control rats also received radioactive ascorbic acid. After the time intervals indicated in figure I animals were decapitated, and adrenal and plasma specimens obtained (5). In some other experiments, further discussed below, the reverse

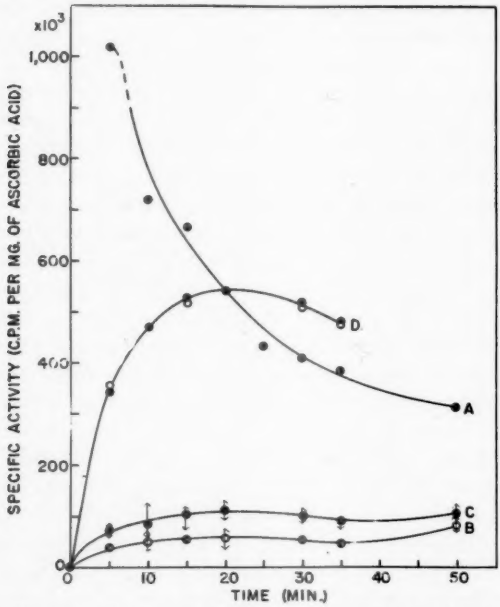

FIG. I. Change with time of radioactivity in ascorbic acid of plasma and adrenal glands of hypophysectomized rats, following administration of radioactive ascorbic acid. Dose: 2.35×10^6 counts per min. (7.04×10^6 counts per min., absolute; 1.00 mg.). Curve A represents the average for ascorbic acid in plasma of all rats. Curve B represents observed values for adrenal ascorbic acid of the control group. Curve C represents observed adrenal values of the test group, which received 3 I. U. of ACTH 45 min. prior to ascorbic-1-C^{14} acid. Curve D represents the combined "true" specific activity values for rapidly-exchanging adrenal ascorbic acid. All specific activities corrected for background and self-absorption, but are not absolute. The upswing at the 50 min. period, which will continue for several hours, is a sign of the by now significant accumulation of radioactivity in the slowly-exchanging fraction of adrenal ascorbic acid.

order was followed, the injection of ACTH following that of radioactive ascorbic acid.

Allowance for the presence of extracellular fluids in calculating amount of radioactivity in adrenal tissue need not be made. *At the most unfavorable point*, 5 minutes after introduction of radioactivity, 1 gm. of adrenal tissue contains about 12×10^4 counts per minute, and plasma 2×10^4 counts per minute per gm. Assuming as much as 20 per cent of the tissue weight to be extracellular fluid, only one-thirtieth could be extracellular radioactivity, which may be neglected.

Results and Discussion

While studying the effect of ACTH on ascorbic acid metabolism in hypophysectomized rats which had received injections of ascorbic-1-C^{14} acid, it was observed that the decrease in adrenal ascorbic acid was not accompanied by decreasing radioactivity. As shown in table 1, radioactivity per gm. of adrenal tissue remained virtually constant, in spite of the considerable decrease in concentration of ascorbic acid found as a result of administration of ACTH in identically-treated non-radioactive rats.

TABLE 1

Effect of ACTH on Adrenal Radioactivity in Hypophysectomized Rats

	Radioactivity in adrenal glands Counts per min. per gm. of tissue		
	a)	b)	c)
	148,000	136,000	103,000
	137,000	158,000	171,000
	127,000	128,000	147,000
Average	137,000	141,000	140,000

Counts corrected for background and self-absorption, but not absolute.
All animals received ascorbic-1-C^{14} acid 30 minutes prior to sacrifice.
a) Control rats, received no ACTH.
b) Received 3 I. U. of ACTH 90 minutes prior to sacrifice.
c) Received 3 I. U. of ACTH 60 minutes prior to sacrifice.
In 3 non-radioactive rats injected with an equal dose of stable ascorbic acid, adrenal glands had an average concentration of 3.27 ± 0.19 mg. of ascorbic acid per gm. of tissue. Another group, identically treated, but receiving 3 I. U. of ACTH 60 minutes prior to sacrifice, had a concentration of 1.93 ± 0.04 mg per gm. (41 per cent decline). Administration of the hormone thus effected an increase in specific activity of adrenal ascorbic acid, since only the concentration of ascorbic acid, but not total radioactivity, was lowered.

Further examination of this problem yielded the data of figure I. This series of experiments involved two groups of hypophysectomized rats. One, the test group, received injections of corticotropin as well as labeled ascorbic acid, while the control group received labeled ascorbic acid, but no hormone. Specific activities of ascorbic acid in adrenal glands and plasma were measured at various intervals after treatment, as indicated. Plasma values

were treated as replicates, there being no evidence for any differences between the two groups in this respect.

Drastic differences were exhibited in specific activities of adrenal ascorbic acid of the two groups. Specific activities of the test group strikingly exceed those of the control group during the entire period of the experiments. This agrees with the data of the preliminary experiment (Table 1). Attention should also be drawn to the fact that both curves which describe the change with time in specific activity of adrenal ascorbic acid attain maxima well before equality with specific activity of ascorbic acid in plasma has been reached. This is inconsistent with unimpeded, continuous exchange of ascorbic acid between plasma and glands.[c] It indicates the operation of processes, such as neogenesis of ascorbic acid in the glands, or incomplete mixing of entering with cellular ascorbic acid. In the absence of such complications, it is required that cellular ascorbic acid not reach a maximal value until equality with the specific activity of ascorbic acid in plasma is reached. That is, the specific activity-time curves for ascorbic acid in plasma and adrenal glands should intersect when a maximum in the latter occurs. It remains to ascertain the discrepancy between anticipated and observed results. The differences in specific activities, between adrenal ascorbic acid of the two groups of animals on one hand, and between adrenal ascorbic acid and ascorbic acid in plasma on the other, may be the consequence of interference from one or more of the following:

1) Presence in adrenal glands of radioactive compounds other than ascorbic acid.

2) Neogenesis of ascorbic acid.

3) Alterations in permeability induced by ACTH.

4) The existence in adrenal glands of two "forms" of ascorbic acid, characterized by

   a) differences in exchangeability, and differing in localization

   b) differences in exchangeability because of difference in state of oxidation

   c) differences in exchangeability due to existence of adrenal "free" and "bound" ascorbic acid.

1) Specific activities of ascorbic acid in adrenal glands were calculated by taking ratios of radioactivity per unit volume of tissue extract to concentration of ascorbic acid per unit volume

of extract (5). If part of the radioactivity in glands of ACTH-treated rats were due to substances other than ascorbic acid, these ratios would be spuriously high. No explanation would be afforded by this for the failure of the specific activity-time curve of adrenal ascorbic acid to intersect the curve for ascorbic acid in plasma. It might explain, however, why test rats had consistently higher specific activities of adrenal ascorbic acid than control rats during the experimental period. The inapplicability of these considerations is shown by the fact that radioactivity in adrenal glands of such rats can be essentially completely identified as unchanged ascorbic acid (4).

2) Adrenal neogenesis of ascorbic acid has been shown to occur at such a low rate, if at all, (5) that it could not possibly be the cause for the disparity between specific activities of ascorbic acid in adrenal glands and plasma at the time maxima occur in curves B and C, figure I, nor between the specific activities of ascorbic acid in adrenal glands of the two groups.

3) Alterations in permeability of adrenal cells might result from administration of ACTH. In the subsequent discussion, it will be shown that this does not appear to be the case. Moreover, it could not account for the great differences in specific activity of ascorbic acid in adrenal glands and plasma at the time maxima occur in curves B and C, figure I.

4) A situation may be visualized in which a part of adrenal ascorbic acid is in rapid exchange with ascorbic acid in plasma, thus acquiring radioactivity, and reaching equality with specific activity of ascorbic acid in plasma, at a rapid rate. If another part of adrenal ascorbic acid were in relatively slow exchange with ascorbic acid in plasma (either directly or *via* the rapidly-exchanging fraction), it may not accumulate an appreciable amount of radioactivity until some considerable time has passed after injection of radioactive material. Under these circumstances, specific activities measured shortly after introduction of radioactivity, representing averages for all adrenal ascorbic acid, would be lower than the "true" values for the rapidly-exchanging form alone. Hence, a maximum in specific activity of adrenal ascorbic acid may well occur before equality with plasma ascorbic acid is established.

(a) The adrenal cortex, consisting of three zones, surrounds a functionally foreign medulla. The possibility must be enter-

tained that differences exist between zones, or between cortex and medulla, in the rates with which ascorbic acid located there exchanges with ascorbic acid in plasma. This could give rise to the phenomena discussed above.

In another paper, it was shown that rapidly- and slowly-exchanging ascorbic acid occurs in erythrocytes (7). This indicates that the two forms may occur together within one cell. Thus, there is no necessity for assuming that the situation may be different in adrenal glands. From the point of view of the solution proposed under (c), it will be inconsequential in any case where within the cortex the two forms are located, since the core of the argument does not hinge on this problem.

The heretofore tacit assumption, that rapidly-exchanging ascorbic acid is located principally in the cortex, was justified by experimental evidence. Pooled adrenal tissues from 4 rats which had received both labeled ascorbic acid and ACTH, and 4 other rats which received labeled ascorbic acid only, were examined. In the former group, the specific activity of ascorbic acid in cortical tissue exceeded that in medullary tissue by 40.0 per cent, in the latter by 25.1 per cent. Medullary tissue preparations contained an appreciable amount of cortical fragments. The relatively low specific activities and lower concentrations of ascorbic acid in medullae, and the relatively small fraction of adrenal tissue represented by medullae, all indicate that only a minor fraction of the radioactivity can be located there. The failure of the medulla to be affected by ACTH cannot be responsible for the constancy of radioactivity per unit weight of tissue (see figure 2), nor for the depression of the observed maxima for adrenal ascorbic acid below expected values.

(b) At this point, it appears fairly certain that there must be a difference in the rates with which two fractions of cortical ascorbic acid acquire radioactivity. Different states of oxidation may, for example, enable two forms to exchange independently, and at widely divergent rates, with corresponding compounds in plasma. The identification of substantially all adrenal radioactivity as ascorbic acid under the conditions of these experiments (4), and the failure of others to discover appreciable amounts of oxidized ascorbic acid in adrenal glands (1), makes it unlikely that this eventuality must be considered further. However, dehydroascorbic acid, the reversibly-oxidized form of ascor-

bic acid, may be considered to be one of the exchanging entities without requiring other than a minor modification of the following interpretation of experimental findings.

(c) In the absence of indications to the contrary, and because of the entirely adequate basis which is furnished for the explanation of all experimental data, it is proposed that the following reaction sequence occurs in adrenalcortical cells:

$$\text{Ascorbic acid (plasma)} \underset{\text{(fast)}}{\leftarrow K_1 \rightarrow} \text{ascorbic acid (adrenal, free)} \underset{\text{(slow)}}{\leftarrow K_2 \rightarrow} \text{ascorbic acid (adrenal, bound)} \xrightarrow[\text{(fast)}]{\text{ACTH}} \text{product(s)},$$

where K_1 and K_2 are the *steady-state* rates of exchange. It may reasonably be assumed that "free" ascorbic acid would exchange at a faster rate than "bound" ascorbic acid with ascorbic acid in plasma. Bound ascorbic acid in adrenal glands has been detected previously, by a different method (2). The existence of a slowly-exchanging fraction of adrenal ascorbic acid, along with a rapidly-exchanging fraction, serves to explain the depression of the observed maxima in adrenal specific activity. By considering the effect of ACTH to be solely directed at the slowly-exchanging fraction, it becomes logical to find the observed, average specific activity of adrenal ascorbic acid greater in hormone-treated than control rats, since the non-radioactive fraction of ascorbic acid is diminished in the former. Similarly, it follows that radioactivity per unit weight of tissue should not be altered by hormone administration, and it is, indeed, not affected (see Fig. II). If the action of ACTH results in formation of products other than reduced ascorbic acid, as indicated by previous experiments (4), no dilution of true specific activity of adrenal ascorbic acid should result when ACTH acts upon the radioactive gland. This, further discussed below, appears also to be true. In addition, in anticipation of data to be published shortly, it follows from the above reaction sequence that a second maximum should occur later in time, at which time equality with the specific activity of plasma should be established, and that ACTH at that time should no longer affect the observed specific activity of adrenal ascorbic acid, but result in diminution of radioactivity per unit weight of tissue, the reverse effect of that shown here for periods shortly after administration of radioactivity. This has been confirmed by additional investigations. In further evaluation of the experi-

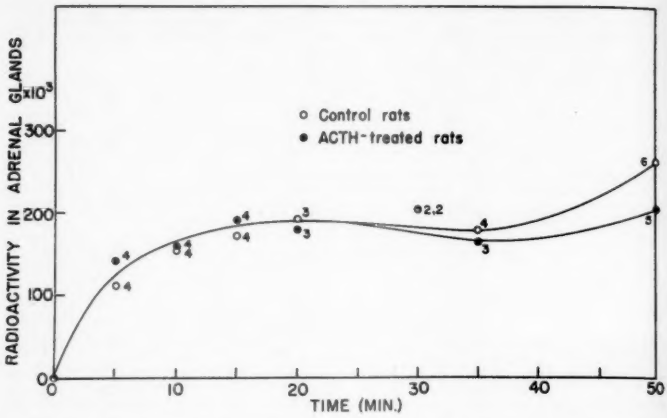

FIG. II. Change with time of radioactivity in adrenal glands. The data are those of curves B and C, figure I, but expressed as radioactivity in c.p.m. per gm. of adrenal tissue. Note the close correspondence between test and control groups, which is absent in figure I. At the later periods, the higher content of adrenal ascorbic acid in the control group causes a somewhat more rapid accumulation of radioactivity by comparison with the test group. Numbers next to experimental points indicate number of rats used in experiment.

mental data, it will be assumed that the above reaction sequence applies.

Calculation of concentrations of rapidly-exchanging and slowly-exchanging adrenal ascorbic acid.—As discussed, the radioactivity of adrenal ascorbic acid, shortly after injection of labeled ascorbic acid, may be considered to reside exclusively in the rapidly-exchanging fraction. This is analogous to the situation in erythrocytes (*7*). At its maximum, the true specific activity of the rapidly-exchanging fraction must attain a value equal to that which the specific activity of ascorbic acid in plasma has at that time. The observed specific activity will be lower because of the presence of the non-radioactive, slowly-exchanging fraction. Hence, to calculate concentrations of rapidly-exchanging and slowly-exchanging ascorbic acid, use may be made of the equality of specific activities of adrenal rapidly-exchanging and plasma ascorbic acid at the maximum of the former. Substitution of appropriate values into equation (*3*) of another paper (*7*) one finds for rapidly-exchanging ascorbic acid for the control group

$$\frac{A_{c,f} = A_{c,\,total} \times r_{c,av.}}{r_{c,f}} = \frac{3.20 \times 58 \times 10^3}{544 \times 103} =$$

0.39 mg. per gm. of tissue and for the test group

$$A_{c,f} = \frac{1.90 \times 144 \times 10^3}{544 \times 10^3} = 0.36 \text{ mg. per gm. of tissue.}$$

These values may be considered to be essentially identical. The concentration of slowly-exchanging ascorbic acid, the difference between total and rapidly-exchanging ascorbic acid, is then 3.20 — 0.34 = 2.86 mg. per gm. of adrenal tissue for control rats, and 1.91 — 0.36 = 1.55 mg. per gm. for the test group. In the latter, ACTH has diminished the concentration of slowly-exchanging ascorbic acid. All other true values for the specific activities of rapidly-exchanging adrenal ascorbic acid may now be calculated by the same equation, solving for $r_{c,f}$ after substitution of the corresponding observed values for the specific activities, all other values being constant and known. The series of points, connected by curve D in figure I, represents the true specific activity-time curve for adrenal rapidly-exchanging ascorbic acid, and is the result of these calculations. As was to be expected, there is no longer any significant difference between test and control rats in this respect. The points have, consequently, been treated as replicates.

The rate of exchange of rapidly-exchanging adrenal and plasma ascorbic acid.—The estimation of the rate of exchange requires the same qualifications mentioned before, and may be accomplished by using equation *(4)* (7). One essential, the necessity of maintaing constant concentration of intracellular ascorbic acid, requires special attention. It is a relatively simple matter to fulfill this condition in control rats, particularly since the experimental periods were so short. But when ACTH was administered to the test rats, the concentration of adrenal ascorbic acid decreased by an average of 41 per cent.

Based on the findings of Sayers et al. (8), it was considered possible to make use of the "trough" or minimum of the ascorbic acid-time curve. The work of Sayers et al. indicated that there should be a time after injection of hormone when the concentration of adrenal ascorbic acid would be relatively constant, although low. This was borne out by preliminary experiments.

TABLE 2

Ascorbic Acid in Adrneal Glands

Time after injection of ACTH (minutes)						
20	35	50	55	60	65	75
		mg. per gm. of adrenal tissue				
1.90	1.96	1.86	2.03	1.63	1.54	1.90
2.22	2.18	1.79	1.80	2.09	1.91	2.03
2.22	2.29	2.05	1.84	2.35	1.30	2.26
	1.22	2.17	1.93	1.53		
	1.76			1.79		
	1.66			2.03		
2.13	1.85	1.97	1.93	1.90	1.58	2.06

Average: 1.91 ± 0.28 (S. D.)

These values represent mg. of ascorbic acid found in adrenal glands of hypophysectomized rats (5 to 6 days postoperatively) at indicated times after injection of 3 I. U. of ACTH. It may be noted that the concentrations remain reasonably constant during these periods.

Average for 80 control rats used in these and related experiments: 3.20 ± 0.38 (S.D.).

Table 2 shows data for the animals actually used in the present work, plus several used in related studies. It appears that for a period from about 25 to 75 minutes after the hormone is introduced, there is little change in concentration of ascorbic acid in adrenal glands of the test group. Unless otherwise indicated, and as mentioned under the methods section, this was chosen as the period during which radioactive ascorbic acid was injected and measurements were made.

A question may now be raised regarding the validity of the comparison between test and control groups as here made. Since measurements on the test group were made more than 45 minutes after injection of ACTH (except on some animals corresponding to data in Table 1), the effect of the hormone may have disappeared at that stage. Thus, while rapidly-exchanging ascorbic acid was found to have equal concentrations in the two groups, it may *have been* affected in the test group at first, but may have had time to be reconstituted. This objection can be shown to be invalid. The data presented in figure I indicate that ACTH may be injected *after* ascorbic acid, without affecting the conclusions: The amount of radioactivity still remains constant, the amount of ascorbic acid decreases, in comparison with test animals.

The last two points on each of the curves of figure I (35 min. and 50 min. periods) represent experiments in which the customary procedure was reversed. Labeled ascorbic acid was ad-

ministered first and, after allowing sufficient time for the rapidly-exchanging adrenal ascorbic acid fraction to become radioactive (15 min.), ACTH was administered. The rats were sacrificed 20 or 35 min. thereafter, *i.e.*, after allowing time for the hormone to act. Control animals received no ACTH. It may be noted that measured values for the specific activity of adrenal ascorbic acid are exactly those which one might have anticipated on the basis of the proposed mechanism. The depletion of adrenal bound ascorbic acid left no recognizable effect on the radioactivity per unit weight of tissue.

The fact that adrenal radioactivity, unlike adrenal slowly-exchanging ascorbic acid, is not affected by ACTH during the periods here considered, has significance in establishing the fate of adrenal ascorbic acid. If slowly-exchanging ascorbic acid were identical with rapidly-exchanging ascorbic acid after release due to ACTH, it should mix with it, and carry outward part of the radioactivity of rapidly-exchanging ascorbic acid. Since it does not, it is indicated that the action of ACTH culminates in a transformation of ascorbic acid. The product seems to be rapidly eliminated from the glands. This is in agreement with other experiments, more direct in nature (4).

Since concentrations of adrenal rapidly-exchanging ascorbic acid and curve D are identical for control and test groups, the rate of exchange between adrenal rapidly-exchanging and plasma ascorbic acid must also be the same for both groups. Graphical integration of equation (*4*) (*7*) between 10 to 35 min. after injection of radioactive ascorbic acid, indicates that the rate K_1 is 2.3×10^{-2} mg. of ascorbic acid per min. per gm. of adrenal tissue. This is a rapid rate and indicative of the copious supply of blood to the glands. It is still insufficient to account for the depleting action of ACTH, even when the flow of ascorbic acid into the glands is disregarded, and the effect visualized as a unidirectional reaction (outward flow only).

One may conclude that rapidly-exchanging ascorbic acid is indifferent to the action of ACTH, and that ACTH affects the cortex in such a way as to liberate slowly-exchanging or bound ascorbic acid. During this process, molecular changes apparently occur, so that the product(s) is no longer identical with ascorbic acid. It may, however, be in the reversibly oxidized form, and thus still be biologically active. The rate of exchange of rapidly-

exchanging ascorbic acid is apparently not affected by ACTH. In consequence, this evidence precludes the participation of changes in permeability of adrenalcortical cells as an appreciable factor in the diminution of ascorbic acid. All factors point toward a much more specific effect of ACTH than might be indicated by simple decrease in retentivity of adrenal cells for ascorbic acid. It is no surprise, under these circumstances, that the kinetics which apply to the exchange of ascorbic acid cannot be made to predict the rate at which depletion takes place.

Summary

A study of the change with time of specific activity in adrenal and plasma ascorbic acid, following administration of radioactive ascorbic acid, disclosed that adrenal tissue appears to contain two forms of ascorbic acid. One of these exchanges rapidly with ascorbic acid in plasma, consequently becoming radioactive soon after injection of labeled vitamin. The other acquires radioactivity much more slowly. It is tentatively suggested that these are identical with "free" and "bound" ascorbic acid, respectively.

The concentrations, and rates of exchange with plasma ascorbic acid, of adrenal rapidly-exchanging ascorbic acid did not differ whether or not ACTH was administered to these hypophysectomized rats.

It appears that changes in permeability are not a likely cause for the depletion of adrenal ascorbic acid following injection of ACTH. The action of ACTH seems specifically and exclusively directed at the slowly-exchanging form, while rapidly-exchanging ascorbic acid is indifferent to the hormone.

It was concluded that the action of ACTH on slowly-exchanging ascorbic acid results in chemical transformations. The kinetics of that process cannot be obtained from the present studies.

The data have been interpreted to indicate the occurrence of a reaction sequence:

$$\text{Ascorbic acid (plasma)} \underset{\text{(fast)}}{\leftarrow K_1 \rightarrow} \text{ascorbic acid } \begin{matrix}\text{(adrenal,)} \\ \text{(free)}\end{matrix} \underset{\text{(slow)}}{\leftarrow K_2 \rightarrow}$$

$$\text{ascorbic acid } \begin{matrix}\text{(adrenal,)} \\ \text{(bound)}\end{matrix} \xrightarrow[\text{(fast)}]{\text{ACTH}} \text{product(s)}.$$

This is in accord with all experimental results.

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[c] The reasonable assumption is made that interstitial fluid does not differ significantly from plasma in regard to concentration and radioactivity of ascorbic acid.

TRANSPOSITION OF GREAT VESSELS, ISOLATED DEXTROCARDIA, COARCTATION OF THE AORTA, PATENT DUCTUS PASSING TO THE DESCENDING AORTA, AND CLEFT PALATE IN A TEN WEEKS INFANT

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Because of successful treatment there has been a large influx of all types of congenital heart disease in recent years. Consequently many bizarre and hitherto undescribed anomalies have been seen in addition to minor variations in arrangement and size of the better known congenital defects. Because of increased experience in this field, and also due to the widespread use of angiocardiography and cardiac catheterization, preoperative and premortem diagnosis is possible in an increasing proportion of patients.

Hamburger (4) was apparently the first to describe the clinical features and particularly the differential cyanosis in a patient with transposition of the great vessels with interruption of the aortic isthmus in which the blood supply to the lower extremities passed through a patent ductus into the descending aorta. Abbott (quoted from Hamburger) describes a similar case and states that she knows of two other cases in "Museum collections." In none of these was there dextrocardia. Taussig (15) discusses Hamburger's case, and also includes a picture of a similar anomaly (p. 484). In her case there was no dextrocardia.

Isolated or rotational dextrocardia is frequent with many types of congenital anomalies and its association with transposition of the great vessels has been described (1). The diagnosis can be strongly suspected from angiographic findings. In the case presented here there was an additional coarctation of the aorta with a patent ductus supplying blood to the descending aorta. From clinical and angiographic findings the basic anomaly was correctly diagnosed prior to death.

CASE REPORT

J.P., W.M., 10 weeks, was born at term. However, the pregnancy had been complicated by intermittent vaginal bleeding and cramping, most noticeable around the 3rd and 5th months. A blood transfusion was given the day before delivery because of placenta previa. In addition, there had been a febrile episode (temperature to 104° F.) during the 4th month diagnosed as influenza and lasting about 5 days.

Birth weight was 6 lbs. 12 oz. A cleft lip and palate were noted. Shortly after birth, the mother noted that the face was more blue than the remainder of the body and frequently, the baby breathed hard and was dyspneic after crying. He also "strangled on his bottle since birth." The baby was fed on evaporated milk, half and half, but at 8 weeks this was changed to Olac because of failure to gain weight. There had been no infection except a "cold" which began about 5 days before admission. The patient was brought to the hospital because of cyanosis, dyspnea, and failure to gain weight.

Family History: Father was 37 years old, alive and well. The mother was 19, and had had a normal child by a previous marriage. Two of the mother's cousins were born with cleft lips while an aunt and uncle have rheumatic heart disease.

Physical Examination: Admission weight was 8 lbs, 2¾ ounces, pulse 140/min; respiration 26/min; B. P. 100/? flush technique. The patient was poorly nourished and developed and in some respiratory distress with substernal retraction. Cyanosis was most marked in the face and upper extremities and only minimal in the lower extremities and trunk, and was increased by crying. The external jugular veins were distended and cardiac dullness was almost entirely on the right side. A loud harsh, grade III-IV systolic murmur was heard over the entire pericardium, but was loudest in the 2nd and 3rd intercostal spaces on the right. Some observers thought that the murmur was continuous. Femoral pulses were of good quality.

Laboratory Data: Hemoglobin 14.4 grams per 100 cc, and hematocrit 47. Other blood analyses and urinanalysis were within normal limits.

Electrocardiogram: Graphs taken with the arm leads reversed and over the right chest, showed flat T waves in I, and this was interpreted as showing right axis deviation and right ventricular hypertrophy.

X-ray Examination: A conventional chest film (Fig. IA) showed that the left heart border was normal in contour, although the aortic knob could not be identified. The right border could not be located as there was a diffuse opacity extending from the mediastinal region to the right chest wall. The borders of this right sided mass pulsated poorly and did not permit differentiation between the heart and a possible adjacent mediastinal mass. The left lung showed some increase in vascularity, but no evidence of infiltration. The right lung was largely obscured by the large central shadow, but the most basilar portion in the costophrenic angle appeared emphysematous. The possibility of a large paramediastinal mass on the right side in addition to a congenital heart lesion was seriously considered.

Angiocardiography: Figure IB-F. Six cubic centimeters of 90 per cent HYPAQUE were injected into the right external jugular vein, and films were exposed at 2 per sec. for 6 seconds. From the superior vena cava, the contrast substance entered a large atrial chamber. Within 1 second thereafter, contrast substance had: (1) entered another atrial structure outlining a sizeable interatrial septal defect, and (2) extended toward the right, outlining a good sized ventricular structure and the aorta. The aorta originated rather far cephalad from the right ventricle and arched sharply toward the left and the midline. The aorta could not be followed beyond the arch in the film made at 2 seconds. There was a delay of 1 second in filling of the descending aorta.

At about the same time there was faint opacification of the smaller pulmonary arteries but the main pulmonary artery was not identified. All of the contrast substance had left the heart within 5 seconds.

The angiocardiogram demonstrated:

1. A large heart with a dextrocardia, rather than a mediastinal mass.
2. A venous ventricle that lay far to the right and emptied entirely into the aorta.
3. Delay in filling the descending aorta.
4. An interatrial septal defect

Because of the differential cyanosis, one could strongly suspect the presence of an aortic obstruction with the distal limb receiving arterial blood from the pulmonary artery thru a ductus. The angiocardiogram confirmed the presence of a transposition with a moderate interatrial septal defect.

Hospital Course: On the 3rd hospital day, following the administration of 0.4 mgm of morphine sulphate, there was a period of apnea lasting 1½ minutes, but the patient quickly responded to artificial respiration and oxygen.

FIG. I. A. Conventional chest film showing a large heart, a bizarre contour with dextrocardia.

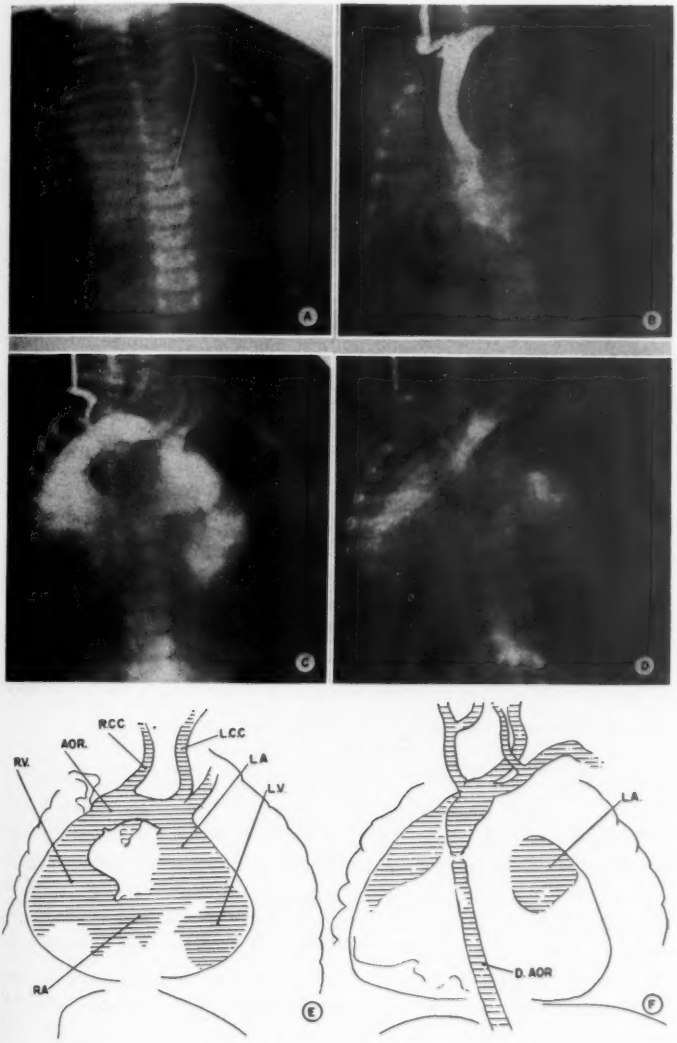
B. Angiocardiogram at 1 second. Contrast substance was injected through the right external jugular vein and entered the venous atrium through a normally placed right superior vena cava.

C. Angiocardiogram at 2 seconds. Contrast substance has passed from the venous atrium into a large ventricular structure and has been ejected into the aorta. The venous (right) ventricle extends almost to the thoracic wall. Contrast material has also passed into the equivalent left atrium and ventricle through an interatrial defect. The brachiocephalic vessels are seen taking origin from the aorta but only a short segment of the descending aorta is seen.

D. Angiocardiogram at 3 seconds. The descending aorta is now visible and some residual contrast substance remains in both right ventricle and left atrium. The pulmonary artery is not seen.

E and F. Sketches of C and D respectively. Aor.—aorta; D. aor.—dorsal aorta; L.A.—left atrium; L. c.c.—left common carotid; L.v.—left ventricle; R.a.—right atrium; R.c.c.—right common carotid artery; R.v.—right ventricle.

Similar apneic episodes followed with increasing severity, and respiratory distress became more pronounced and the patient died on the 10th hospital day.



The Anomalies Present and an Interpretation

The heart and great vessels

Consider first the deviation of the heart at right angles to the body axis with the apex to the right (Fig. II). This is the original

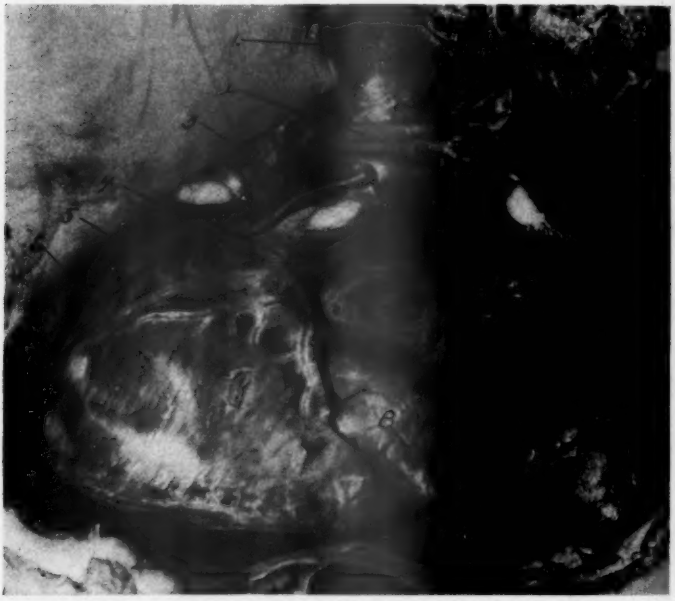


FIG. II. Ventral view of the heart in situ: (1) Larynx, (2) Innominate vein, (3) Aortic trunk, (4) Right coronary artery, (5) Right ventricle, (6) Left ventricle, (7) Right auricle, (8) Left auricle, (9) Left lung.

orientation of the heart when herniated into the extraembryonic coelom in early somite stages. Normally this is corrected after retraction of the herniated loop. However the full correction with the apex to the left depends upon a clockwise spiraling of the ventricular muscle whose tonus pulls the interventricular septal margins into a counterclockwise spiral. Such correction only partially occurred in this heart. The coronary arteries do not form a spiral on its surface. The second deviation to the right must have followed the establishment of the vena cavae at about the end of the second month. The incident force involved

is not evident. It is true that the right lung is displaced and suppressed eliminating one lobe, reducing its weight (66g. on the right to 88g. on the left), and dragging its root to the left (Fig. 3). Cause and effect are not clear. The right ventricle is smaller than the left indicating that from early stages there was less blood poured into it. Since the right atrium is huge much of the venous blood must have been shunted through the 10 mm. foramen ovale to the left (Fig. IV). Since the capacities of the two ventricles

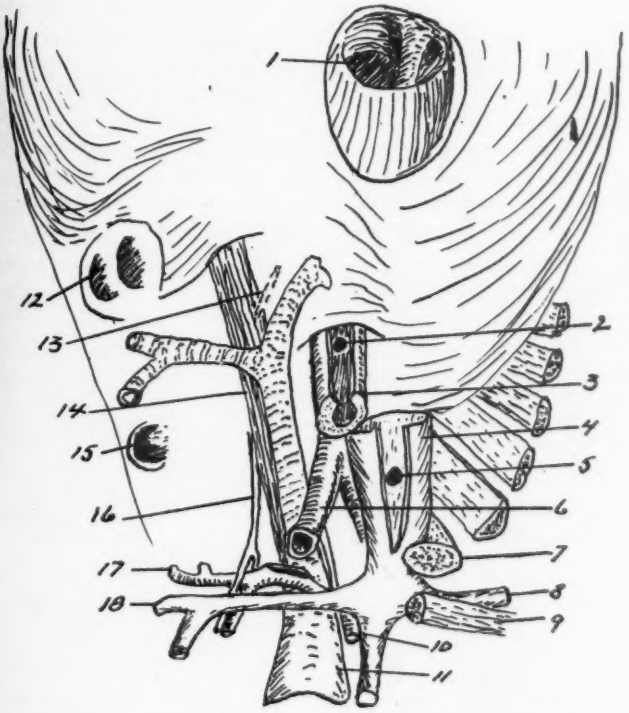


FIG. III Cranial view of the great vessels in the thorax (1) Inferior vena cava at the hepatic division, (2) Opening of the aortic arch into the descending aorta, (3) Ductus arteriosus, (4) Superior vena cava, (5) Opening of the azygos vein, (6) Right aortic arch and right subclavian artery, (7) First rib, (8) Right subclavian vein, (9) Clavicle, (10) Right carotid artery, (11) Larynx, (12) Pulmonary veins, (13) Location of left pulmonary vein, (14) Esophagus, (15) Left pulmonary artery, (16) Vagus nerve, (17) Left subclavian vein.

differ greatly the length of systole for the left ventricle was longer and oxygenated blood was injected into the right ventricle through the persistent 5 mm. foramen in the septum (Fig. V). This was the only oxygen supply to the right ventricle. The persistence of both of these openings also goes back to the second month of development.

Axis deviation has peculiar effects on the location of entrances to superior and inferior vena cavae (Fig. VI). These vessels are located in normal relations to the thorax but the inferior vena cava (23 mm.) has eight or more times the sectional area of the superior vena cava. The axis of these vessels is transverse to the heart. The left coronary vein is small (Fig. IV) and lies in the floor of the inferior vena cava. All other venous relations in the chest are normal.

The arteries are decidedly not normal. There is complete transposition of the trunks so that the aorta rises from the right ventricle. The larger pulmonary trunk arising deeper from the left ventricle, quickly branches into the right pulmonary arch and ductus arteriosus and a left pulmonary arch. Passing forward the aortic trunk gives rise to the left subclavian and the carotid arteries and then curves dorsally as the right arch (Fig. III). This arch at the level of the second rib gives off the right subclavian artery and continues as a thinner walled channel to the small

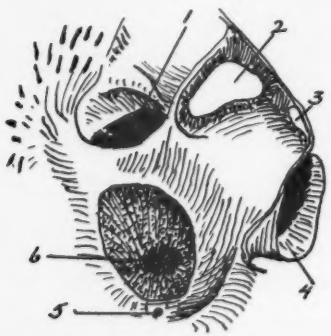


FIG. IV. Interatrial septum from the left side: (1) Foramen ovale (10 mm.), (2) Left pulmonary artery (lung 88g.), (3) Right pulmonary artery lung 61g.), (4) Inferior vena cava (22 mm.), (5) Left coronary vein, (6) Left A-V valves.

FIG. V. Interventricular septum from the right: (1) Aortic valves, (2) Interventricular foramen with membranous flap.

opening in the floor of the ductus arteriosus. The anoxic condition of the carotid body may have been a factor in the ventricular hypertrophy (70 g.). The angle of entrance would preclude much regurgitation from the ductus to the right arch. The persistence of the right arch again indicates that the anomaly was determined by the end of the second month. The ductus then supplies

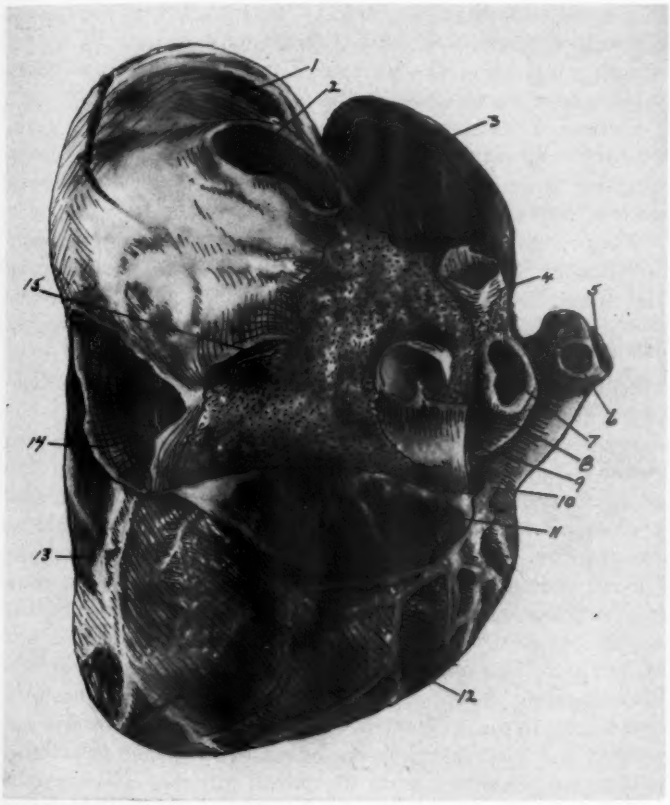


FIG. VI. Dorsal view of the heart on its own axis. The heart weighs 70 g. and is 60 mm. wide: (1) Left auricle, (2) Left pulmonary vein, (3) Right Auricle, (4) Ductus arteriosus, (5) Aortic opening to the head, (6) Right aortic arch, (7) Ductus arteriosus, (8) Left Pulmonary artery, (9) Superior vena cava, (10) Right coronary artery, (11) Part of right atrium, (12) Right Ventricle, (13) Left Ventricle, (14) Inferior vena cava. (15) Right pulmonary vein. Stippled area shows pericardial attachment.

the trunk and lower limbs with oxygenated blood. None of the viscera below the diaphragm show any anomalies.

Returning to the coronary arteries we find them coming from the aortic trunk even though that trunk has been transposed and its valves derived from the right side (Figs. II and VI). This indicates that the heart wall was being supplied by oxygen-poor blood. This may well have been the immediate cause of death. It also indicates that the origin of the coronary arteries is not determined by the position of the valve cushions but by the descending septum of the truncus. The origins of the coronary arteries have been discussed elsewhere (2).

Sections of the lungs show them to be normal in detailed structure and fully expanded. A few alveoli contain occasional monocytes but there is no pneumonic process. The walls of the vessels show no hypertrophy.

The present case differs from anything in the atlas of Abbott, or Taussig (15), or the extensive series of cases studied by Harris and Faber (1939).

Etiology of cardiac anomalies

Cardiac anomalies are so lethal that genetic studies are hampered. Familial concentrations of such defects have been noted by many authors (8). Others (17) on the basis of twin studies with almost complete lack of placental data contended that there is no genetic factor involved. Since the genes are part of the mechanism producing anomalies, the number of such anomalies should increase as surgery saves these individuals to reproductive age. Cardiac anomalies are also the functional result of prior anomalies and this introduces variation. For instance if blood islands are inadequate heart development lags. Because of this fact evolutionary explanations of such defects are not much help in understanding. A correlation with faulty implantation has been noted (13). In the present case we know that placenta previa was present and early bleeding had occurred. So little attention is paid to the placenta at birth that much valuable information is lost. Interference with cellular respiration produces cardiac and other anomalies. A variety of experimental agents have been used (3). Cellular anoxia from maternal disease in early pregnancy may also be a factor.

Reversed symmetry if complete would lead to no physiological

difficulty. Isolated reversal causes incoordination (8). The mechanism of double axis formation by which monozygotic twins become independent provides for all degrees of influence of one upon the other leading to partial mirror imaging. This fact confuses the uniform action of a gene on both members, especially in the choice of alternate paths from a primarily bilateral pattern. Twin studies therefore are not good criteria of gene influence on deviations from symmetry. Some degree of mirror imaging is very common between identical twins. This may involve hair whorls, order of teething, scrotal descent, handedness or visceral asymmetries. In armadillo embryos it extends to the whole epidermal pattern.

Etiology of harelip and cleft palate

Harelip and cleft palate result from the failure of the lateral primordia of lip and palate to complete their fusion with medial primordia. In the palate this is accomplished both by growth medially and by the raising of a vertical plate from the oral cavity. In the present case (Fig. VII) the right upper lip is completely cleft and the left shows a notch at the point of fusion. The cleft continues back to the pharynx. The left palate is normal

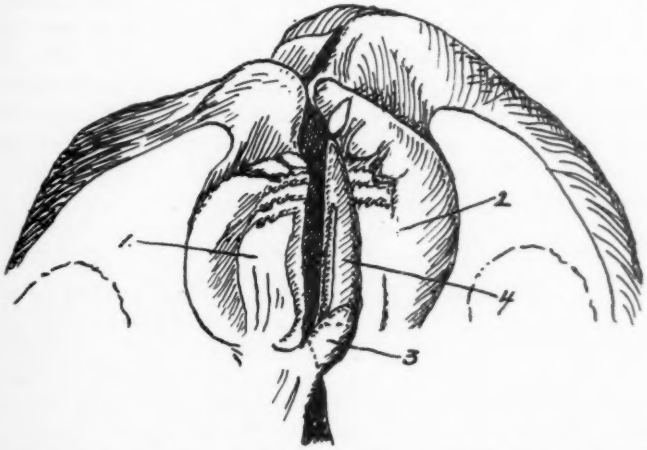


FIG. VII. Ventral view of cleft palate: (1) Shortened right palate with small tooth germs, (2) Fully elongated left palate with large tooth germs, (3) The right uvula lying over the left but not fused to it, (4) Internasal septum fused to left palate.

with well developed tooth germs. The right nearly as wide has failed to contact the septum though its vertical plate has been elevated and differentiated. Uvulae are present on both. The right palate however has not elongated to match its mate and the tooth germs are smaller. This retardation must have begun at least by the third month. It is not related to general anoxia of the head because differential anoxia did not exist until birth.

A careful probing into the family history often discloses similar anomalies. Despite the fragmentary history in this case there were two known cases of harelip in the immediate family. Genetic factors, generally recessive, play a background role making the anomalies possible in the presence of general or local factors operating on the primordia at the time of their final determination. This activity may express itself as mitotic flares, active migration, or simply change of state. The literature on the genetics of cleft palate is profuse, the most conclusive being the study of monozygotic twins. Lehman and Ritter (19, 39) showed that in fifteen pairs with harelip eleven were concordant and four discordant. It is difficult to prove monozygocity, unless one accepts similarity as proof, and this explains some of the discordants. Harelip may appear in only one of cojoined twins (11, 12). Cojoined twins may not necessarily come from a single placenta and may not be monozygotic. If they are we find that they have a common circulation (13). Others (17) are still stating that monozygotic twins may come from separate placentas. Reed (19, 36) found that malimplantation correlated with harelip in mice. Harelip in mice has appeared as a mutant whose frequency and expressivity can be altered by respiratory or nutritional factors (9).

Summary

A ten weeks old male died with cyanosis of the head and arms. Angiocardiograms defined; dextrocardia, transposed trunks and aortic coarctation. Autopsy disclosed a large heart, transverse and to the right of the body axis. Aortic trunk and both coronary arteries arise from the small right ventricle. This received only venous blood via the caval veins except for foramina in both atrial and ventricular septa. The large pulmonary trunk arises from the left ventricle. Both pulmonary veins enter the left atrium. The right aortic arch persisted and developed coarctation between

the origin of the right subclavian artery and the very large ductus arteriosus which continued as the descending aorta. Lungs are histologically normal. Complete right cleft palate was present and a genetic basis is indicated. Death was probably caused by cardiac hypoxia.

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CALLING ATTENTION TO

OCTOBER, 1957

Basic Biology: SH Bartley surveys fatigue & inadequacy (*Physiol Rev* 37/301/57). EB Beckett & GH Bourne note increased phosphatase activity in dystrophic muscle (*Science* 126/357/Aug/23/57). MWH Bishop & CR Austin report on mammalian sperm (*Endeavor* 16/137/57). PW Brian & JF Grove discuss gibberellic acid as plant growth agent (*ibid* p 161). CL Coman & Co analyse strontium-calcium movement from soils to humans (*Science* 126/485/Sept 13/57). RD Dripps edits *The Physiology of Induced Hypothermia* (Nat Acad Sci, Washington, DC, 57, 460 pp, $3.5). O Eichler & A Farah offer *Morphine und Norphinannlich wirkende Verbindungen*, 12th Vol, *Erganz Hndb exper Pharmakologie* (Lange & Springer, Berlin W35, 376 pp, 72DM). LR Fitzgerald surveys cutaneous respiration in humans (*Physiol Rev* 37/325/57). E Frommel & P Gold report on action sites of rauwolfia alkaloids on sleep center (*Acta Pharmacol Tox* 13/345/57). JH Gaddum & HO Schild edit symposium on drug antagonism (*Pharmacol Rev* 9/211–268/57). MI Gluckman & Co show iproniazid (monamine oxidase inhibitor) causes cerebral synaptic inhibition like serotonin (*Science* 126/448/Sept 6/57). E Guerra & B Gunther note relations of organ weight & function to body weight (*Acta Physiol Latinoamer* 7/1/57). W Heubner & Co continue studies on oxidation of hemoglobin (*Arch exper Pharmakol* 230/421/57) & short appreciation of Heubner appears (*ibid* 231/1/57). JC Krantz & Co note convulsive action of hexafluoro-diethyl ether (*Science* 126/353/Aug 23/57). HA Krebs analyses control of metabolic processes (*Endeavor* 16/125/57). DK Lee edits symposium on bioclimatology (*Fed Proc* 16/603/57). A Lipschutz reviews *Steroid Homeostasis, Hypophysie & Tumorigenesis* (Heffer, Cambridge, 57, 92 pp, 30s). NF Maclagan edits symposium on physiological & clinical aspects of livers (*Brit Med Bull* 13/75–149/57). SP Narikashvili edits Festschrift to Ivan Beritashvili including ED Adrian's "Transmission of information from olefactory organ," HW Magoun on specific brain mechanisms, CJ Herrick on analytic & integrative nervous function, WH Gantt on cardiac conditioned reflex under reserpine, & E Bozler on smooth muscle function (Acad Sci Georgian SSR, Tibilisi, 56, 583 pp). WDM Paton discusses histamine release (*Pharmacol Rev* 9/269/57). CP Swanson reviews *Cytology & Cytogenetics* (Prentice-Hall, Engelwood, NJ, 57, 596 pp, $14).

Clinical: M Allgower & Siegrist edit *Verbrennungen: Pathophysiologie, Klinik, Therapie* (Lange & Springer, Berlin W35, 57, 333 pp DM 88). SP Bessman & Co find deficiency in 5-hydroxytryptamine formation in hepatic coma (*Proc Soc Exp Biol Med* 95/502/57). PF Halora & Co find low protein intake in anemia causes high incidence of gastric ulceration (*Ibid* p 238). RG Hunter & Co find papain & bromelein (proteolytic enzyme from pineapple stem) relaxing to uterus (*Amer J Obs Gyn* 73/867, 875/57). JH Moyer & Co note chlorothiazide ("Duiril") a potent non-toxic orally effective diuretic (*Proc Soc Exp Biol Med* 95/529/57). PJ & L Nestel find increased urinary

aminoacid concentration in erythroblastosis & suggest abnormal nitrogen metabolism in kernicterus (*Med J Austral* 1/908/June 29/57). JA Parr & Co discuss cellular features of lupus erythematosis (*ibid* 900, 905, 911). DM Rathman reviews *Vegetable Oils in Nutrition* with reference to unsaturation (Corn Products Refining Co, NY, 57, 70 pp, free). NS & TN Stern reveal *The Bases of Treatment* (CC Thomas, Springfield, Ill, 57, 187 pp, $6).

General: FJ Cole publishes early extension of Harvey on circulation by Henry Power (1623–1668) (*J Hist Med* 12/291/57). JG Cozzins succeeds in shocking in *By Love Possessed*, Harcourt Brace, NY, 57, 570 pp, $5). HA Davidson offers a *Guide to Medical Writing* (Ronald Press, NY, 57, 338 pp, $5). P Dansereau tackles *Biogeography* (Ronald Press, NY, 57, 405 pp, $7.5). EW Hall considers *Modern Science & Human Values* (Van Nostrand, Princeton, NJ, 57, 483 pp, $8). M Kerker considers Sadi Carnot (1796–1832) & start of themodynamics (*Sci Month* 85/143/57). A Koestler is provocative in *Reflections on Hanging* (Macmillan, NY, 57, 231 pp, $4.5). H Kreighbaum notes *Where Doctors Meet Reporters* (NY Univ Press, NY, 57, 119 pp, $2.5). G Langford gives *Alias O Henry: A Biography of Wm Sidney Porter*, remarkable Texan & Ohioan (Macmillan, NY, 57, 294 pp, $5). SB McKinley is solid in *Woodrow Wilson: A Biography* (Praeger, NY, 57, 284 pp, $4.5), while AL & JL George psychoanalyse him in *Woodrow Wilson & Colonel House: A Personality Study* (J Day, NY, 57, 356 pp, $6), & H Hoover will laud him in new study. EH Mudd & A Krich edit *Man & Wife: A Source Book of Family Attitudes* (WW Norton, NY, 57, 317 pp, $5). WF Thompson asks why don't scientists admit they're human/ (*Sat Rev*, Sept 7/57, p 44). CJ Wiggers criticizes Harvey's report on circulation in Harvey Lecture (*Circ Res* 5/335/57).

NOVEMBER, 1957

Preclinical: NI Arlashchenko & GM Erdman show cortically implanted electrodes give constant irritation (*Sechenov J Physiol USSR* 43/18/57). JR Brobeck describes neurothermic control of hunger (*Yale J Biol Med* 29/565/57). HM Felton edits McLaughlin Conference on *Host-Parasite Relationships* (CC Thomas, Springfield, Ill., 57, 269 pp. $7). S Garattini edits *Psychotropic Drugs* (D VanNostrand, Princeton, NJ, 57, 650 pp, $14). B Glass goes in pursuit of a gene (*Science* 126/683/Oct 11/57). PJ Morgane & AJ Kosman find hyperphagia & weight gain after bilateral amygdalectomy (*Nature* 180/598/Sept 21/57). RF Nigrelli edits symposium on current ideas on spontaneous generation (*Ann NY Acad Sci* 69/255–376/57). CC Pfeiffer & Co note stimulant action of 2-dimethylaminoethanol, possible precursor of brain acetylcholine (*Science* 126/610/Sept 27/57). G Popjak & E LeBreton edit *Biochemical Problems of Lipids* (Interscience, NY, 57, 509 pp, $11). H Roskova & J Vanecek find streptolysin intraventricularly to cats gives symptoms of chorea (*Science* 126/700/Oct 11/57). BF Skinner describes experimental analysis of behavior & includes effects of drugs (*Amer Sci* 45/343/57). K Soehring analyses organization of autonomic nervous system (*Med Klin* 52/1129/June 28/57). WS Spector edits *Handbook of Biological Data* & also *Handbook of Toxicology II Antibiotics* (WB Saunders, Phila., 57, 584 pp,

$7.5; 264 pp, $5). P Talalay analyses enzymatic paths in steroid metabolism (*Physiol Rev* 37/362/57). K Tanaka & H Mishima show antiphlogistic action of morphine is mediated by adrenergic nerves (*Yonago Acta Med* 2/55/57). CH Thienes & Co report laxative, hemostatic & toxic effects of alginic acid preparations (*Arch Internat Pharmaco* 111/167/57).

Mundane Satellite Publications: M Kramer & Co study penicilinase formation (*Acta Physiol Acad Sci Hung* 12/125, 133, 139/57). A Orosz & Co show $H_3P^{32}O4$ is absorbed from nasal mucosa to accumulate in hypothalamus & basal ganglia (*ibid* 11/177/57). I Pataky & Co study inhibition after electroshock (*ibid* p 45). J Porszasz & Co note adrenolytic action of aminoketones (*ibid* p 29). L Tokacs studies circulation & metabolism in hypoxia (*ibid*, p 67, 189, 197). K Kiersz & Co find reserpine causes blood sugar rise (*Acta Physiol Polon* 8/125/57). J Ryzewski & Co find hypothermia stops visceral reflexes (*ibid* p. 201).

Clinical: H Behrendt analyses clinical aspects of *Chemistry of Erythrocytes* (CC Thomas, Springfield, Ill., 57, 242 pp, $6). JBC Baker & Co find quick recovery of isolated hearts arrested by perfusion of K-citrate lmgm/cc Locke's sol (*Lancet* 2/555/Sept 21/57). A Briskier suggests *Cardio-Charting: Universal Method of Recording Heart Auscultation* (Macmillan NY, 57, 58 pp, $5). JH Burn edits report on "Fluothan" (chlor-brom-trifluoro-ethane), noninflammable inhalation anesthetic (*Brit Med J* 2/479/Aug 3/57). EG Dimond compiles data on *Digitalis* (CC Thomas, Springfield, Ill, 57, 272 pp, $7). P Gyorgi & LE Holt Jr edit Symposium on Pediatric Disorders & Nutrition (*Amer J Clin Nutr* 5/491–565/57). FB Peck edits reports on toxicity of sulfonylureas as antidiabetic drugs (*Diabetes* 6/1–92/57). R Platt edits symposium on Physiology & Pathology of Kidney (*Brit Med Bull* 13/1–70/57. AK Reynolds discusses *Morphine & Allied Drugs* (Univ Toronto Press, Toronto Press, Toronto 5, 57, 393 pp, $10). ES Schneidman & Co considers *Clues to Suicide* (McGraw-Hill, NY, 57, 227 pp, $5.5). AL Welsh writes *The Dermatologist's Handbook* (CC Thomas, Springfield, Ill, 57, 427 pp, $10). F Wroblewski notes serum lactic dehydrogenase changes in malignancy (*Amer J Med Sci* 234/301/57).

General: RF Ewer analyses ethological concepts related to neurophysiology (*Science* 126/599/Sept 27/57). WB Gallie asks what makes a subject scientific (*Brit J Phil Sci* 8/118/57). AS MacNalty describes public health influence of W Harvey (1578–1657) (*Roy Soc Health J* 77/324/57). JM Murtagh dares anyone to *Cast the First Stone* (McGraw-Hill, NY, 57, 307 pp, $4.5). M Neiburger shows weather modification to stop smog is impractical (*Science* 126/637/Oct 4/57). HM Mark opens symposium on giant molecules (*Sci Amer* 197/81–216/Sept 57). JR Paul describes medical mission to Russia (*Sci Month* 85/150/57). M Polyani discusses problem solving (*Brit J Phil Sci* 8/89/57). Tributes to George Sarton (1884–1956) are edited by IB Cohen (*Isis* 48/283–350/57). J Schubert & RE Lapp discuss *Radiation: What It Is & How It Affects You* (Viking, NY, 57, 314 pp, $4). F Schoenberner is heroic in *You Still Have Your Heads: Excursions from Immobility* (Macmillan, NY, 57, 247 pp, $4). E Weeks edits *Jubilee: 100 Years of The Atlantic* (Little-Brown, Boston, 57, 745 pp, $7.5). G Williams considers *The Sanctity of Life & the Criminal Law* (AA Knopf, NY, 57, 291 pp, $5).

DECEMBER, 1957

Preclinical: BW Agranoff & Co show meprobamate in liver metabolized with 20% unchanged slow urinary excretion (*Proc Soc Exp Biol Med* 96/261/57). Amer Physiol Soc publishes Iowa City Fall Proceedings in *The Physiologist* (1/6-92/57). RA Beatty discusses *Parthenogenesis & Polyploidy in Mammalian Development* (Cambridge Univ Press, 57, 144 pp, $3). A Berglas proposes inducing premature division of cancer cells as means of causing their death (*Cancer: Nature, Causes, Cure;* Pasteur Inst. Paris, 57, 154 pp). EM Bickoff & Co isolate estrogen, coumestrol, from forage crops (*Science* 126/969/Nov 8/57). E Bueding & C Swartzwelder survey recent anthelmintics (*Pharmacol Rev* 9/329/57). HJA Dartnall reviews *The Visual Pigments* (Wiley, NY 16, 1957, 216 pp, $6). SJ Foley analyses *Physiology & Biochemistry of Lactation* (CC Thomas, Springfield, Ill., 57, 162 pp, $4). BN Halpern & Co give *Physiolpathology of the Reticulo-Endothelial System* (CC Thomas, Springfield, Ill., 57, 325 pp, $9). G Hoyle analyses *The Nervous Control of Muscle* (Cambridge Univ Press, 57, 160 pp, $3). WA Krivoy finds chlorpromazine & reserpine inhibit spinal reflexes (*Proc Soc Exp Biol Med* 96/18/57). E Marciniakowna finds adrenalin shortens blood coagulation by increasing thrombokinetic activity (*Acta Physiol Polonia* 8/17/57). J Mills & Co survey substituted ethylene glycols for anti-convulsant action & pick phenaglycodol (2–chlorophenyl–3–methyl–2,3–butane–diol) as best (*Proc Soc Exp Biol Med* 96/100/57). K Nados studies molecular form of drugs in relation to action (*Proc Hung Physiol Soc Acta Physiol* Suppl XI, 80pp/57). MW Noall & Co note endocrine control of amino acid transfer (*Science* 126/1002/Nov 15/57). RL Noble considers hormonal regulation of tumor growth (*Pharmacol Rev* 9/367/57). HM Peck & Co show gibberellic acid has no mammalian toxicity (*Science* 126/1064/Nov 22/57). CC Porter & Co find ceruloplasmin oxidation of serotonin (*ibid* 1014/Nov 15). AF Rasmussen & Co show increased susceptibility to herpes in mice subjected to avoidance-learning stress or restraint (*Proc Soc Exp Biol Med* 96/183/57). G Sato & Co show molecular growth requirements of single mammalian cells (*Science* 126/961/Nov 8/57). PA Shore & Co indicate serotonin & norepinephrine inactivation by monoamine oxidase (*ibid* 1063/Nov 22).

Clinical: H Behrendt offers *Chemistry of Erythrocytes: Clinical Aspects* (CC Thomas, Springfield, Ill, 57, 242pp, $6). JW Bellville & GM Attura describe servo control of general anesthesia (*Science* 126/827/Oct 25/57). JB Campbell & Co report regeneration of adult mammalian spinal cord 30 days after transection (*ibid* 929/Nov 1/57). H McIlwain gives *Chemotherapy & the Central Nervous System* (J&A Churchill, London, 57, 238pp, 45s). T Rosebury in tribute to WJ Gies (1871–1956) challenges dentistry (*Science* 126/1056/Nov 22/57). RHS Thompson & EJ King, edit *Biochemical Disorders in Human Disease* (J&A Churchill, London W1, 57, 522 pp, 90s). J Tuckman & M Lavell find self-classification reliable on feeling old or not old (*Geriatrics* 12/666/57). JM Tyberghein & RH Williams find hypoglycemic action of phenethyldiquanide related to anaerobic glycolysis with lactic acid increase (*Proc Soc Exp Biol Med* 96/29/57).

For Holidaze: E Bergler reveals *The Psychology of Gambling* (Hill & Wang,

NY, 57, 212pp, $3). J Cohen reviews subjective estimation of probability (*Sci Amer* Nov 57, p128). GW Gray reviews biological organizers (*ibid* p79). M Mead points to more vivid utopias (*Science* 126/957/Nov 8/57). OHP Pepper is swell as *Old Doc* (Lippincott, Phila 57, 247pp, $3.75). LC Ray & RA Kirsch suggest finding chemical records by digital computors (*Science* 126/314/Oct 25/57). RS Ropp considers *Drugs & the Mind* (St Martin's Press, NY, 57, 308pp, $4.50). AJ Simon raves over *The Noble Grapes & the Great Wines of France* (McGraw-Hill, NY, 57, 180pp, $15). J Thorwald goes over *The Century of the Surgeon* (Pantheon, NY, 57, 321pp, $6).

At Year's End: A Eliot goes over *Three Hundred Years of American Painting* (Random House, NY, 280pp, 1957, $13.50). G Goodman says there's *A Time for Paris* (Doubleday, NY, 57, 288pp, $3.75). M Hilliard offers *A Woman Doctor Looks at Love and Life* (Doubleday, NY, 57, 256pp, $4). HI Marrou gives *A History of Education in Antiquity* (Sheed & Ward, NY, 57, 422pp, $7.50). PN Medawar considers *The Uniqueness of the Individual* (Basic Books, NY, 57, 220pp, $4).

C. D. Leake

PROCEEDINGS, TEXAS BRANCH OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

Abstracts of Some of the Papers Read at the Fall Meeting

NOVEMBER 22–23, 1957

TRINITY UNIVERSITY, SAN ANTONIO, TEXAS

Growth Studies on the Cotton Root Rot Fungus, *Phymatotrichum Omnivorum*. I. The Effect of Hydrogen-ion Concentration and Oxygen in Liquid Culture.

R. J. Hervey, Texas Agricultural Experiment Station.

The fungus causing cotton root rot in alkaline soils belongs to the Fungi Imperfecti. It produces non-functional conidiospores in soils; none on artificial media. *Phymatotrichum omnivorum* grows irregularly, exhibiting prolonged lag-phase. To induce consistently rapidly growing viable-sporulating cultures, H-ion and oxygen-effect were selected for initial study.

The fungus is environmentally adjusted to alkaline soils, but grows extensively on liquid ph-4-initially-adjusted liquid media. Growth is poorer on similar pH-7 media. Ten-day mycelial growth in pH-4-media weighed 515 mgs.; in pH-7-media 281 mgs.

Abundant oxygen is requisite for rapid extensive growth, but vigorous shaking markedly curtails growth. Initiation of growth on floats is faster and greater than in shallow liquid. Early stagnant growth is better in 25 ml.-volumes than in 50 ml.

H-ions in metabolized ph-4-adjusted media decrease with incubation due to NO_3-utilization and cation accumulation. pH-7-adjusted-media increases in H-ions during early incubation due to organic acid production.

Effects of Hemophilus Pertussis Vaccine on Anaphylaxis in Mice.

W. E. Harris, Lt., USAF; John D. Fulton, Lt. Col., USAF (MSC), Department of Microbiology, School of Aviation Medicine, USAF, Randolph AFB, Texas.

A micro-hematocrit technique for the quantitation of the extent of sublethal anaphylactic response in mice has recently been reported by the authors. The use of *Hemophilus pertussis* vaccine (HPV) as an adjuvant in studies on anaphylaxis in mice is well known. This study was designed to determine whether or not the inclusion of HPV in the sensitizing and/or challenge antigen would modify the anaphylactic response as measured by hematocrit change. It was observed that HPV when contained in the sensitizing antigen would cause an enhancement of the anaphylactic response as measured by the hematocrit method, whereas HPV administered in the challenge antigen did not cause an enhancement of the response.

Preliminary Study of the Effects of X-Irradiation on the Antibody Combining Capacity of Human Proteins. William G. Glenn and Iowa W. Marable, Department of Microbiology, School of Aviation Medicine, USAF, Randolph Air Force Base, Texas.

Lyophilized human plasma, immune globulin, serum albumin, and pooled normal human serum were exposed *in vitro* to x-irradiation up to 200,000 roentgens. These antigens were then titrated with homologous rabbit antisera by the alpha procedure and the extent of reactivity measured by the Photron-reflectometric or light scattering method. Analyses were done in duplicate at 2 hours and 7 days post-irradiation. Only in irradiated serum albumin was any appreciable difference in affinity for homologous antibody observed. Such difference was apparently transitory. Serum agar analyses failed to confirm protein dimerization following irradiation as reported by other workers. It would seem that, except for serum albumin, when human proteins at high concentrations are exposed to large doses of x-irradiation the antigens, as measured by their affinity for homologous rabbit antibody, are not altered.

Effect of Cortisone Injections on Complement Titers in Guinea Pigs. L. Renshaw and R. K. Guthrie, North Texas State College, Denton, Texas.

The C'4 component of rat and guinea pig Complement has been observed to increase following antigenic stimulation, whereas nonantigenic substances caused no consistent change in titer. Such findings indicate that the C'4 component may be associated with the immune response.

To test this supposition, groups of guinea pigs were injected with cortisone, cortisone plus egg white antigen, and egg white antigen alone. One uninjected group served as controls.

Cortisone injected animals exhibited no consistent change in C'4 titer while those receiving egg white showed the expected increase in C'4 titer. The animals injected with both cortisone and egg white antigen exhibited a significant decrease in C'4 complement titers. These results indicate that the increase in C'4 titer after antigen injection is a part of the immune response.

Experience with the Zymosan Assay of Properdin. R. A. Finkelstein, L. M. Roch, and S. Edward Sulkin, The University of Texas Southwestern Medical School, Dallas, Texas.

The present work confirms the usefulness of the zymosan assay of properdin proposed by Pillemer, *et al.* (*J. Exp. Med.* 103: 1, 1956). The assay is a fairly simple procedure provided one starts with suitable biological reagents, RP and R3. This problem has been resolved to the point of practicality by the use of pools of large numbers of human sera for the preparation of reagents. There are still some limitations because of apparent incompatibilities of sera of certain species with the reagents used. Since many laboratories are experiencing difficulties in reproducing the properdin assay, we felt it was important that confirmation of the technique be presented.

On the basis of limited study, it appears that the McNall assay technique (*Proc. Soc. Exp. Biol. Med.* 94: 399, 1957) does not provide reliable data on the properdin content of human and animal sera.

Mixtures of Coagulase Positive Staphylococci from Clinical Sources. E. D. Rosenblum and J. L. W. Jackson, The University of Texas Southwestern Medical School, Dallas, Texas.

In a study of over 200 routine clinical isolations of coagulase positive staphylococci, 14 proved to be distinct mixtures differing in pigmentation or degree of pigment. The components of the mixture also differed in phage type and/or to one or more antibiotics. In most instances pigment differences were detectable on Staphylococcus Medium No. 110 but not on blood agar. The clinical significance of mixed infections were not determined, but means of isolation and implications for bacteriophage typing and antibiotic sensitivity testing were discussed.

The Stimulatory Effect of Egg Yolk Dialysate on the Growth of Mycobacteria. J. O. Norman and R. P. Williams, Baylor University College of Medicine, Houston, Texas.

Dialysates prepared by dialysing egg yolk against Beck-Proskauer medium or distilled water stimulated the growth of certain mycobacteria including H37Rv. The stimulatory activity is not completely lost by autoclaving or exposure to extremes in pH, but can be eliminated by serial dilution. Dialysates prepared with contaminated egg yolk are not active.

Concentrated dialysates behave as if lipid materials were present in rather large concentrations. Chemical tests for cholesterol were negative. Oleic acid used in various concentrations did not cause as marked a response of the organisms as did the egg yolk dialysate. Oleic acid would not stimulate growth at all in the absence of albumin while dialysates are active in the absence of albumin.

Variants of *Bacillus Anthracis* Produced by Exposing Spore-Contaminated Bone Meal to Dry Heat. Reuben D. Wende and Kenneth L. Burdon, Department of Microbiology, Baylor University College of Medicine, Houston, Texas.

In testing dry heat as a sterilizing agent for raw bonemeal which had first been steam-sterilized, then deliberately contaminated with anthrax spores, variants of *Bacillus anthracis* were recovered on culturing the bonemeal after heating at temperatures and times short of complete sterilization.

Variants, non-virulent for mice, of three colonial types, including pink-pigmented varieties, were repeatedly recovered from bonemeal contaminated with spores from two virulent strains of *Bacillus anthracis*, after heating at 120° C, 140° C, and 150° C for 30 minutes. Another strain yielded a still-virulent culture after 140° C for 30 minutes, and a non-virulent variant after 160° C for 30 minutes. Morphological changes in the variants varied from slight to marked, but characteristic biochemical reactions of *B. anthracis* were retained.

Characteristics of Ozone Activity Against *Escherichia coli*. Irving Davis, Captain, USAF (MSC), Department of Microbiology, School of Aviation Medicine, USAF, Randolph AFB, Texas.

Continued attempts to investigate the quantitative lethality of ozone were described. Ozone was quantitatively produced by a Welsbach water-cooled

corona discharge ozonator using dry, clean oxygen. The ozonated-water solutions constituted stock solutions for the biological studies. Analyses for ozone in aqueous solutions were carried out spectrophotometrically. The lethal effects of ozone on the microbial population as reflected by survival-time experiments showed that (1) death of cells occurred within the first minute or less of contact with ozone, longer cell-ozone contact periods not producing increased death within the given population, *i.e.*, an "all or none" phenomenon, and (2) the lethality process operated in both the ultraviolet-resistant and ultraviolet-sensitive strains of *E. coli*, the process being quantitatively more lethal in the latter strain.

Enteric Infections Occurring in Rhesus Monkeys by *Shigella Flexneri* Resistant to Normal Antibiotic Therapy. Charles E. Craft, B.S.; Roland B. Mitchell, Ph.D., Department of Microbiology, School of Aviation Medicine, USAF, Randolph AFB, Texas.

An epidemic of Shigellosis occurring in a colony of monkeys failed to respond to the usually effective therapeutic agent, chloromycetin. Tests for the susceptibility to drugs of the causative agent, *Shigella flexneri*, revealed that the antibiotic, Polymyxin B, and the chemical compounds, furadantin and furazolidone, were effective *in vitro*. In addition, tests on various drug combinations were evaluated. Utilizing these laboratory results, the monkeys were placed on a regimen of furazolidone. Marked improvement of the animals was observed within 5 days, with complete recovery of all animals within 20 days. All cultures were negative for Shigella after 14 days. It is believed that this is the first recorded incident where furazolidone was used as the therapeutic agent in monkeys.

Demonstration of Particulate-Bound Pigment in *Serratia Marcescens*. W. W. Taylor and R. P. Williams, Baylor University College of Medicine, Houston, Texas.

Strain Nima cells were disrupted in a high-speed mechanical shaker, and then subjected to step-wise centrifugation. After treatment at 50,000xg for ½ hour, the sediment and supernatant both were pigmented. Centrifugation at 105,000xg for 1 hour resulted in a colorless supernatant and a pigmented pellet. Resuspension of the latter in distilled water yielded a clear but pigmented liquid whose spectrum at pH 5.4 showed maxima at 260 and 495, a shoulder at 530, and a minimum at 425 mμ. The analytical ultracentrifuge pattern of the material showed three peaks, one coinciding with the pigment. Preparations from non-pigmented cells grown at 37° C showed only one peak and no pigment. Pigment could not be dialyzed from the particles. The latter contained protein, carbohydrate, lipid, RNA, and DNA.

A Water-Soluble, Diffusible Pigment from a Strain of *Serratia Marcescens*. R. P. Williams, D. Hawkins, Jr., and W. W. Taylor, Baylor University College of Medicine, Houston, Texas.

Cells of strain 125 are colorless. Pigment is red and diffuses into the medium. Biochemical tests established the strain as *S. marescens*. Pigment is ex-

tracted from the medium with activated charcoal. Following elution with glacial acetic acid, pigment was further purified by continuous paper electrophoresis. The pigment can be dialyzed, is soluble in water, but insoluble in acetone, chloroform, ethanol, ether, and petroleum ether. No fractions can be separated from the pigment by either acid or basic paper electrophoresis. A highly-fluorescent compound can be separated in an isopropanol-HCl solvent system. The acid, base and neutral spectra of purified pigment are similar with maxima at 280 and 550, and a minimum at 430 mμ.

Isolation of Protoporphyrin Ix from the Catalase of *Micrococcus Lysodeikticus*. S. Miller and R. P. Williams, Baylor University College of Medicine, Houston, Texas.

A method was presented for isolating protoporphyrin IX from a one liter volume of organisms. The microorganism was chosen because of its catalase content and susceptibility to lysozyme. Catalase was purified by a modification of the procedure of Herbert and Pinsent. Catalase was treated with HCl and acetone in order to obtain haematin. Iron was removed from haematin by refluxing with powdered iron and formic acid. Protoporphyrin IX was purified by extraction with chloroform followed by partition chromatography on cellulose powder using 2.6 lutidine-water as the developing solvent. The Rf value and absorption spectrum were comparable to those of an authentic sample of purified protoporphyrin IX. This procedure provides a method for studying porphyrin metabolism in bacterial cells by the use of isotopes.

Recent Laboratory Findings on Epidemic Influenza. J. E. Grimes, A. Canales, and T. D. Sullivan, Texas State Department of Health, Austin, Texas.

An epidemic of Asian type influenza in Texas was confirmed by this laboratory. Virus isolation confirmed eighteen cases of the disease. Four of these were in Austin, three in Texas A. & M. College, two in Houston, and one in each of the following cities: Tyler, Seguin, Waco, Richmond, Arlington, San Benito, Texarkana, Plainview, and San Antonio. Type specific hemagglutination inhibition tests confirmed eighty-seven cases of Asian influenza in forty-two different cities.

Recent Laboratory Findings on Aseptic Meningitis. C. R. Gabriel, T. D. Sullivan, and J. V. Irons, Division of Laboratories, State Department of Health, Austin, Texas.

This summer specimens with histories indicating aseptic meningitis were received. Five viral agents from stools and one from a throat wash were isolated in monkey kidney tissue cultures. They were neutralized by ECHO type 9 antiserum and were not pathogenic for baby mice until they had been propagated in monkey kidney cells. Paired blood samples on one patient showed a four-fold increase in neutralizing titer against ECHO type 9 virus. The isolates originated from Port Arthur, Freeport, Plainview, and Tyler.

Neutralizing Antibodies Following Vaccination Against Rabies. Alice Conklin and J. V. Irons, Division of Laboratories, State Department of Health, Austin, Texas.

A series of three doses of H E P Flury vaccine failed to produce measurable neutralizing antibodies, and a booster dose did not produce measurable neutralizing antibodies. A single booster dose of H E P Flury vaccine given after previous experience with Pasteur or Semple vaccine produced a startling rise in neutralization index, even though the dose was small or given years ago. The immunity produced by H E P Flury vaccine may not be entirely measured by neutralizing antibodies. Other mechanisms may play a part, such as interference, especially if the virus multiplies in the body. Embryonated duck vaccine produced a definite booster effect when used after a short series of the duck embryo vaccine.

Inactivation of the Herpes Virus by the Properdin System. Richard A. Finkelstein, Rae Allen, and S. Edward Sulkin, The University of Texas Southwestern Medical School, Dallas, Texas.

In a previous report from this laboratory, it was shown that a strain of herpes simplex virus was inactivated by a factor in normal rat serum with properties similar to the properdin system. Use of the Pillemer assay of properdin in this laboratory made it possible to establish a firmer relationship between the serum inhibitor and the properdin system.

Seven strains of herpes simplex virus from diverse sources were susceptible to the properdin system. Serum depleted of properdin had no effect but became virucidal upon the addition of purified properdin; complement being necessary for the reaction. Resistance to properdin is not readily induced by repeated passage and selection of survivors.

The possible role of the properdin system in resistance to manifestations of herpes virus infection was discussed.

Evidence of Viral Lipotropism in Bat Rabies. S. Edward Sulkin, Philip H. Krutzsch, Craig Wallis, and Chansoo Kim, The University of Texas Southwestern Medical School, Dallas, Texas.

The purpose of the present study has been to examine the pathogenetic sequence in experimentally infected insectivorous bats with the view to learning something of the possible mechanism by which these animals may serve as persisting reservoirs for the virus in nature. The results suggest that the rabies virus is capable of proliferation in the interscapular brown fat of the Mexican free-tailed bat following intramuscular inoculation. These studies in addition to including the rabies virus among those (poliovirus and certain Coxsackie viruses) which exhibit lipotropism suggest that this may prove to be a characteristic feature of infection with "hardy" viruses.

Studies on Turkey Ornithosis by Means of Fluorescein-Labelled Antibody. P. Donaldson, D. E. Davis, J. R. Watkins, and S. E. Sulkin, The University of Texas Southwestern Medical School, Dallas, Texas, and Texas A&M College, College Station, Texas.

Tracheal swab materials taken on the seventh day from ten turkeys artificially infected with ornithosis virus were inoculated upon yolk sac explants

on flying coverslips in roller tubes. The cover slips were removed after 48 hours and stained with fluorescein-conjugated antibodies against ornithosis. Eight of the specimens gave fluorescent developmental forms of ornithosis. Ten day specimens from the two turkeys yielding negative results were cultured and also found negative. There was indication that cloacal specimens might be at least as valuable as tracheal specimens for tissue culture. Direct smears of the seventh day tracheal swabs showed fluorescein-antibody stained material some of which appeared to be bacteria, but developmental forms in intact cells were not seen.

The Cytological Response of Embryonated Eggs to Normal and Hepatitis Infected Mouse Livers. B. A. Rubin and H. A. Kemp, Baylor University College of Medicine, Houston, Texas.

Liver homogenates and serum from mice with viral hepatitis were able, under proper conditions, to cause a cytological response in the allantoic fluid of suitably inoculated embryonated hen eggs. Although the sera of normal mice of the same strain were not capable of eliciting this response, extracts of normal mouse livers could produce the effect. In no case was the cytological effect serially transferable.

No amount of serum or liver extract could produce an effect nearly as large as that obtained with influenza virus. In general rather large amounts of infected serum or liver extracts are required to produce any statistically valid level of effect. This effect with murine hepatitis material could be correlated with the presence of enzymes in the serum of diseased animals which were present in both normal and diseased livers at all times, but not found in normal mouse sera.

Direct Hemagglutination of Rabbit Red Blood Cells by Lymph Node Pus in Cat Scratch Fever. Major C. D. Graber, MSC, Chief, Bacteriology Branch, U.S.A. Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Texas.

While attempting to sensitize mammalian erythrocytes with a 1:5 dilution of epitrochlear lymph node pus obtained from a clinically adduced case of cat scratch fever from a patient with a positive skin test to CSF antigen it was discovered that this material directly hemagglutinated rabbit red cells. This type of hemagglutination, which was not characteristically of the viral type, was inhibited by human serum from another case of cat scratch fever and by hyperimmune serum produced in a rabbit to positive pus sensitized red cells. Absorption of both type sera with pus from a positive skin test case and lymph node material from another case—not cat scratch fever—removed inhibiting agglutinin in the case of the former only. An attempt to elute this hemagglutinating agent for agglutination of freshly washed rabbit cells was not successful.

CHANGES IN ERYTHROCYTE AND PLASMA ENZYME ACTIVITY ASSOCIATED WITH NEOPLASIA[a]

(Preliminary Note)

EDWARD BRESNICK AND WILLIAM C. LEVIN[b]

During an investigation of the metabolism of erythrocytes of patients with various types of malignancies, it was found that the activity of certain enzymes was altered. Elevation of the serum lactic dehydrogenase activity has been reported in mice with either spontaneous, induced, or transplanted tumors (4), as well as in patients with certain neoplastic diseases (2, 3, 10). However, we were unable to find any previous report of the influence of neoplasia upon erythrocyte transaminase activity. The results of an investigation of this enzyme activity are presented in this preliminary report.

Venous blood samples were obtained from normal adults (medical students and nurses), and from patients with various neoplastic diseases (carcinomas, lymphomas, Hodgkin's disease). Heparin was used as an anticoagulant. After the plasma was aspirated from the centrifuged blood samples, the leucocyte and platelet layers were removed. The erythrocyte layer was resuspended in 0.9 per cent saline and recentrifuged. This procedure provided an essentially leucocyte-free preparation, containing negligible amounts of plasma.

An aliquot of the erythrocyte preparation was hemolyzed in fifty volumes of distilled water. The protein concentration of the plasma and of the hemolysate was determined by the biuret method (9). Plasma and erythrocyte lactic dehydrogenase (LD) were determined according to the method of Wroblewski and LaDue (10). Glutamate-oxalacetate transaminase activity (GOT) was determined according to Steinberg's modification (7) of Karmen's method (5). The activity was expressed as units per milligram protein, where one unit equals a decrease in optical density (at 340 mμ) of 0.001 per minute per milligram protein (representing the rate of oxidation of reduced diphosphopyridine nucleotide). This method of expression was empirically found to result in less variation.

Wroblewski and LaDue (10) reported the normal LD activity to range between 260–850 units per ml. serum. Assuming a normal protein concentration of 70 mg. per ml. serum, this range would correspond to 3.7–12 units per mg. protein. Our data of normal individuals, therefore, agree substantially with those of Wroblewski and LaDue.

In addition, Wroblewski *et al.* (10) stated that the whole blood hemolysates were about 100 times as active as the serum. Assuming that the erythrocyte comprises 40 per cent of the total volume of blood and that hemoglobin (15 gms. per 100 ml. blood) comprises more than 90 per cent of the total proteins of the erythrocyte, then, 1 ml. of erythrocytes contains approximately 410 mg. protein. This corresponds to an erythrocyte lactic dehydrogenase activity of 25,400 units per ml. red blood cells. Our normal results agree substantially with those red blood cell values of Wroblewski and LaDue.

The data are presented in Table 1. Although the plasma and erythrocytes of different neoplastic diseases were investigated, the averages of the enzymatic activities show considerably higher values than that of the controls (normals). At this stage, no attempt was made to determine the differential effects of various types of neoplasia upon the enzyme activity, but the individual results were tabulated to illustrate the various neoplasias employed. The lactic dehydrogenase activity in the normal erythrocytes was 62.0 ± 4.0 units/mg. protein, whereas in neoplasia, it was 82.5 ± 4.6, an increase of 33 per cent ($t = 7.1$). Similarly, in the plasma of patients with neoplastic disease, the activity was 8.24 ± 0.94 against 4.25 ± 0.20, of the controls ($t = 9.6$).

The transaminase activity was also elevated in the erythrocytes of patients with neoplasia: 2.11 ± 0.20 units as compared to 1.22 ± 0.08 of the controls, which corresponds to an increase of 80 per cent ($t = 5.1$).

In view of the fact that elevated rates of anaerobic glycolysis have been observed in intact tumors growing *in situ* (1), as well as in tissue slices of tumors (8), these data were not unexpected. The high plasma LD activity could represent "leakage" from the tumor. An analogous situation exists in clinical and experimental myocardial infarction, wherein high serum LD values have also been demonstrated (10).

In patients with neoplasia, there is, furthermore, an increase

TABLE 1

Effect of Neoplasia Upon Plasma and Erythrocyte Enzyme Activity

Patient	Lactic Dehydrogenase Erythrocyte Units per mg. Protein	Lactic Dehydrogenase Plasma Units per mg. Protein	Transaminase Erythrocyte Units per mg. Protein	Diagnosis
Normal	62.0±4.0(13)*	4.25±0.20(10)*	1.22±0.08(11)*	
PATIENTS WITH NEOPLASIA:				
G.	99.3	12.6	2.20	Cancer of lung
B.	95.5	4.26	2.70	Cancer of lung
D.	87.2	6.84	1.50	Metastatic cancer
B.	75.7	6.88	2.90	Carcinoma
N.	73.6			Carcinoma
L.	92.0	7.15	1.60	Lymphoma
A.	69.7	9.22	2.17	Metastatic cancer
T.	63.8	5.68	1.70	Hodgkin's disease
W.	98.5	12.1		Hodgkin's disease
J.	79.8			Hodgkin's disease
K.	93.0	9.37		Lymphosarcoma
M.	69.2			Monocytic leukemia
J.	75.7			Monocytic leukemia
N.			1.69	Lymphoma
K.			2.33	Skin cancer
L.			2.13	Cancer of face
B.			3.19	Lymphosarcoma
Average	82.5±4.6*	8.24±0.94*	2.11±0.20*	
Change	+33%	+94%	+80%	

* The values are means ± standard error. The number of individual determinations is indicated within the parentheses.

in the erythrocyte LD and GOT activities. The possibility that this, too, is due to "leakage" from the tumor is very small because of the relative impermeability of the erythrocyte membrane. Such a substance could have its origin either in the neoplastic tissue itself, or it could arise from other sources as an expression of disordered metabolism imposed upon all organ systems by the neoplastic disease. Further support of this hypothesis is provided by the results obtained by Reid *et al.*, who observed the higher rate of phosphate loss from erythrocytes of mice with large mammary tumors than from normal animals (6). They attributed this phenomenon to an alteration of the enzyme systems in the erythrocyte due to a substance produced by the tumor. These results are of particular interest in view of the relationship existing between phosphate metabolism and glycolysis.

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A COMPARISON OF THE TOXIC EFFECTS OF VARIOUS LOCAL ANESTHETIC DRUGS ON HUMAN CILIATED EPITHELIUM *IN VITRO*[a]

GUNTER CORSSEN AND CHARLES R. ALLEN[b]

Since Koller described the local anesthetic properties of Cocaine in 1884 (7), continued efforts have been made to find similar and more effective compounds for use in local anesthesia. The search for more potent and at the same time less toxic agents stimulated the development of methods for testing these drugs in order to evaluate their usefulness and safety for clinical use.

Recently Hirschfelder and Bieter (6) reviewed the numerous techniques used for determining or comparing the activity of local anesthetics. Most of the methods described by these authors require the use of laboratory animals. The skin of the frog or the cornea of the rabbit or guinea pig usually serve for testing the effect of new agents for topical anesthesia. Muscle nerve preparations or isolated nerves for investigating the effectiveness of the drug in conductive anesthesia are also taken from these animals. All of these test methods can give only a general idea of the potency and toxicity of a drug under investigation since it is very difficult to transfer the results obtained to clinical usage in man.

The intradermal wheal, performed in man, attempts to overcome these difficulties but again we deal with a number of variable conditions such as different pain thresholds in different individuals.

In searching for more sensitive and more objective methods for comparison of the toxic properties of local anesthetic agents we decided to utilize recent developments in tissue culture techniques and study the effects of these drugs on the cell.

The proposal to expose cultured tissue to various local anesthetics and to observe the effect of these drugs on cell activity is based on the assumption that local anesthetics act as protoplasmic poisons (1). The drug apparently affects the metabolism of the cell resulting in a disturbance of its function. This can be

either transient and completely reversible or can result in permanent damage to the cell structure with irreversible injury to its activity depending upon the chemical structure of the local anesthetic, its concentration and the amount and rapidity of absorption by the cell. Ciliated epithelium appeared to be most valuable for such cytotoxic studies since the ciliary beat can easily serve as an index of cellular activity. In the past, several authors have been interested in the effects of various chemicals on ciliary action (2, 3, 5, 10, 11, 21, 22, 23). The experiments were usually performed with excised strips of tracheal, esophageal or pharyngeal mucosa of various animals (frog, rabbit, rat, horse, ox, sheep). The gill of the oyster has also been used for this purpose. Recently studies have been reported on the action of various drugs on ciliary activity when topically applied to the upper respiratory tract of man (8, 13, 14, 16). The effect of changes in temperature (17) and the influence of different ions and changes in pH on ciliary action have also been investigated (4, 9, 12, 13).

In 1936 and 1939 Proetz and Pfingsten (18, 19) described a method for cultivating ciliated nasal mucosa of the guinea pig fetus. In 1949 a tissue culture study of human ciliated nasal epithelium was reported by Rose, Pomerat, and Danes (20).

Since July 1956 successful cultivation of human tracheal and bronchial epithelium has been performed in the Tissue Culture Laboratory of The University of Texas Medical Branch, Galveston. When electrolyte balance and other physiological factors such as osmotic equilibrium are present cultures of human respiratory epithelium maintain a steady state for 15 to 20 hours. Increased or decreased strength or rapidity of ciliary beat or disturbance of coordination of ciliary motion following exposure of the cells to the drugs under study may, therefore, be interpreted as a cytotoxic effect of the local anesthetic agent. The observation of such tissue with phase-contrast photomicrography and, particularly, with cinematography seemed to offer ideal conditions for observing and recording the results of such investigations.

Methods and Materials

During general anesthesia, tissue was obtained from the tracheal and bronchial mucosa of man by means of a small caliber biopsy punch without the use of local medication. The average size of the specimens was approximately

5 to 8 mm. square yielding 3 to 6 explants each about 2 to 3 mm. square. These biopsies were placed in Gey's balanced salt solution (BSS) immediately after removal from the trachea or bronchus. In the course of this study 1004 explants from 66 different patients were cultured and examined. The explants were set up in hanging drop slides as described by Rose, Pomerat, and Danes (20) as follows: Under sterile precautions each individual explant was embedded in a clot formed by the addition of one part of chicken plasma containing 1000 gamma of Neomycin per milliliter to one part of embryonic extract and four parts of human ascitic fluid.

Ciliary action which ceases immediately after transferring the tissue into the test tube with Gey's balanced salt solution usually was seen again within 6 to 8 hours after being embedded in a plasma clot. It reached maximum activity within 2 to 3 days and was kept about 10 to 12 days. Usually the outgrowth of an epithelial sheet from the explanted tissue began with the reactivation of ciliary motion (Fig. I). Frequently such sheets showed a tendency

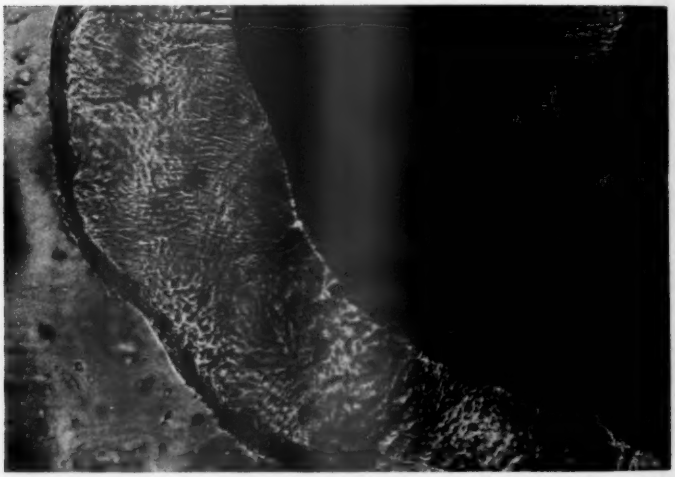

FIG. I. Dense bar represents the explanted fragment of human bronchial mucosa. Outgrowth of the epithelial sheet began 24 hours after incubation at 37° C. Photograph was made on the 4th day. Magnification: 67 X.

to lose their continuity so that pieces of epithelial fragments often curled to form little globes or balls which rotated by means of their actively beating cilia (Fig. II). As soon as such rotating globes appeared the whole explant was transferred into a perfusion chamber equipped with an inlet and an outflow tube (15). After thoroughly sealing the cover glass carrying the explant to the roof of the chamber by using melted beeswax and paraffin, Gey's balanced salt solution at room temperature was perfused through the system. This did not disturb the activity of the rotating spheres. Local anes-

thetic solutions to be perfused through the chamber were prepared by dissolving the crystals of each drug in Gey's BSS since, as a rule, commercially available local anesthetic solutions, tablets or powder contain preservatives and binding substances which generally have a deleterious effect on the cell in culture. The effect on the ciliary action was observed with either phase-contrast, time lapse cinematography at a rate of four frames per minute, or by scanning at a rate of sixteen frames per second.

The type of response of the epithelial globe following exposure to the various local anesthetic agents at different concentrations is believed to represent the toxic effect on the cell. An increase of ciliary action appears to be the result of a stimulatory effect which can persist for a measurable length of time, or may

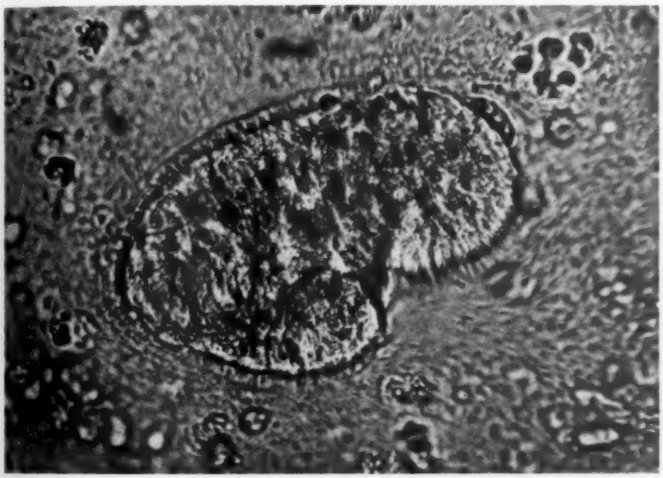

FIG. II. Rotating globe of human tracheal epithelium. Photograph was made on the 8th day of incubation at 37° C. Magnification: 442 X.

only be a transient phenomenon. A depressant action results in a decrease of ciliary activity resulting in the slowing of rotary speed and finally stoppage of this activity. The depressant action is either reversible indicating a temporary, non-deleterious effect on protoplasmic activity or, irreversible in which case the reperfusion with Gey's BSS does not, or only partially leads to revival of ciliary function.

It is possible to determine for each local anesthetic under observation the concentration which

a. does not affect ciliary activity,
b. causes persistent stimulation,
c. results in initial stimulation with subsequent decrease of activity,
d. stops ciliary action, but with re-perfusion with BSS results in revival of the tissue,
e. arrests ciliary action with permament cell injury and re-perfusion with BSS fails to re-activate ciliary beat.

A comparison of five clinically useful local anesthetics—Novocaine, Xylocaine, Cocaine, Pontocaine and Nupercaine as to their effect on human ciliated epithelium in tissue culture is presented in Table 1.

TABLE I

RESPONSE OF CILIARY MOVEMENT OF HUMAN RESPIRATORY EPITHELIUM TO LOCAL ANESTHETIC DRUGS

Response of Ciliated Cell	Novocaine in percent	Xylocaine in percent	Cocaine in percent	Pontocaine in percent	Nupercaine in percent
No Effect	0.05	0.01	0.05	0.01	0.001
Persistent Stimulation	0.1-2.0	0.1-2.0	0.1-0.5	0.015	0.003
Initial Stimulation with subsequent Depression	5.0-10.0	5.0	1.0	0.025	0.03
Stoppage of Ciliary Activity Reversible	20.0	20.0	10.0	0.1	0.05
Stoppage of Ciliary Activity Irreversible			20.0	0.15	0.1
Vibration	1.0-20.0	0.5-20.0	0.5-20.0	-	-

Novocaine and Xylocaine act in a similar manner with regard to their effects on the activity of cultured ciliated epithelial cells. Although neither drug affects ciliary function at concentrations of .01 per cent (Xylocaine) to .05 per cent (Procaine) they both cause persistent stimulation at a concentration of 0.1

per cent. A stoppage of ciliary activity and complete recovery after removing the agent and re-perfusion with BSS immediately after the ciliary arrest is complete is observed with Novocaine and Xylocaine at concentrations up to 20 per cent. Occasionally this effect could be found with Xylocaine at concentrations as low as 10 per cent.

Novocaine and Xylocaine have a remarkably wide range of discernible effect on cell activity without causing permanent damage to the cell. At concentrations ranging from 1.0 per cent to 20 per cent with Novocaine and 0.5 per cent to 20 per cent with Xylocaine these agents cause a peculiar vibration of the whole tissue usually in conjunction with signs of a disturbance of the coordinated beat.

Pontocaine and Nupercaine affect cultured ciliary epithelium at considerably lower concentrations as compared to Novocaine and Xylocaine. Persistent stimulation results from concentrations as low as 0.015 per cent with Pontocaine and 0.003 per cent with Nupercaine. Exposing the tissue to Pontocaine 0.15 per cent or Nupercaine 0.1 per cent is followed by a rapid decrease and subsequent stopping of ciliary action. The cells fail to revive when Gey's BSS is re-perfused. There is a considerably smaller margin of safety with these drugs with regard to their least effective concentrations as compared to those concentrations causing irreversible damage to the tissue. No vibrating of the tissue is observed with Pontocaine or Nupercaine.

Cocaine seems to occupy a position between the Novocaine-Xylocaine group and Pontocaine-Nupercaine group. Cocaine at 0.1 per cent causes continuous stimulation and a 10 per cent solution arrests ciliary action usually with incomplete recovery. Cocaine at 20 per cent renders the cell damage irreversible.

Whether or not the exposure to the local anesthetic drug dissolved in BSS might change the osmotic equilibrium significantly and affect ciliary action this way requires further study. It seems questionable that the usually instant response of ciliary movement as observed with all local anesthetics at effective concentrations can be related to osmotic disturbances. There is a strong possibility, however, that delayed effects on ciliary action occurring, for example, half an hour or later following the perfusion of the drug can be partially explained by this mechanism. The same holds for changes in pH of the culture medium. A determination

of the hydrogen ion concentration for the different local anesthetics at various concentrations revealed variations ranging from 7.8 (Dibucaine 0.01 per cent, Tetracaine 0.015 per cent) as the most alkaline to 5.6 (Cocaine 20 per cent, Xylocaine 20 per cent) as the most acid solutions. Between these extremes of pH are all other local anesthetic solutions tested and a correlation could not be made between ciliary activity and the pH of the solutions.

Summary

Cultured ciliated respiratory epithelium of man, observed microscopically, offers an objective method for testing the effects of local anesthetic agents upon the cell. The ciliary beat which remains undisturbed when the explant of respiratory epithelium is transferred into a balanced salt medium serves as an index of cell activity. With exposure to different local anesthetic drugs at various concentrations ciliary activity undergoes changes such as acceleration, deceleration or arrest, or disturbance of coordination of the ciliary beat. The effect is either reversible or results in permanent cell injury. The intensity with which local anesthetics affect cell function as reflected by ciliary activity seems to be directly proportional to the anesthetic and toxic properties of the agent.

This method has been used for a comparative study of Novocaine, Xylocaine, Cocaine, Pontocaine, and Nupercaine. For each of these local anesthetics the concentration which either did not affect cell activity, or resulted in acceleration, deceleration or stoppage of any visible cellular activity could be determined.

The low toxicity and the remarkably broad spectrum of effectiveness of Novocaine and Xylocaine upon the cultured cell is significant. Both drugs first affected the tissue at concentrations of 0.1 per cent and failed to produce permanent cell injury at concentrations up to 20 per cent.

Pontocaine and Nupercaine revealed a considerably higher toxicity to the cells. The margin of activity without causing permanent damage to the cells ranged from 0.015 per cent to 0.1 per cent for Pontocaine and 0.03 per cent to 0.05 per cent for Nupercaine. Cocaine occupies a position between the Novocaine-Xylocaine and the Pontocaine-Nupercaine group.

A peculiar trembling or shaking of the whole tissue was seen

with Novocaine, Xylocaine, and Cocaine at certain concentrations which are believed to represent a disturbance in the mechanism of ciliary coordination.

Conclusion

Comparative studies of the effects of the various local anesthetics upon cell activity using cultured ciliated respiratory epithelium of man revealed minimal toxic effects of Novocaine, Xylocaine and to a certain extent Cocaine at clinically useful concentrations. Pontocaine and Nupercaine proved to be considerably more toxic and rendered cellular activity irreversible at concentrations commonly used for producing regional anesthesia in man.

NOTES

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CANCER CHEMOTHERAPY WITH PLANT EXTRACTS

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Screening of plant extracts for possible anticancer effects has continued since our last report (Taylor *et al.*, 1956). A total of 388 tests were made involving 181 species of plants[b] for anticancer activity. The plant collecting data and some information as to chemical constituents is available (Wall *et al.*, 1945a, 1945b, 1955). Some of the plants have been tested for antibacterial activity (Frisbey *et al.*, 1954, 1955; Gottshall *et al.*, 1955; Lucas *et al.*, 1955a, 1955b).

Experimental Procedure

The extracts were prepared as shown by Wall *et al.*, 1954c. The plant fractions were tested against both egg cultivated tumors and tumors transplanted in mice. The methods used have been described (McKenna *et al.*, 1954; Taylor, 1953a, 1953b; Taylor *et al.*, 1952, 1956).

Yolk sacs of embryonated eggs were inoculated through the air sac on the 4th day of incubation with a saline suspension of dispersed tumor tissue. A C3H mouse mammary carcinoma, especially adapted to growth by this type of cultivation, was used. The test extract was injected over the area vasculosa of the tumor bearing eggs on the 10th or 12th day of incubation, or 6 to 8 days after the eggs were inoculated with the mouse tumor. The effect of the plant fraction was based on weight differences in the tumors and embryos of the experimental eggs as compared with the controls which developed after treatment with the extract.

For tests against tumors cultivated in mice, a dba mouse mammary carcinoma was used. This tumor is known under the designation of RC mammary adenocarcinoma and has been used in screening programs in other laboratories (Tarnowski and Stock, 1955). Mice with transplants of this tumor were given one injection of the test material at or near the tolerated dosage. The effect of the plant extract was evaluated on the basis of the average weight of the tumors from the treated as compared with the tumor size in the control groups for a 10-day test period. Data on body weight changes were also recorded.

Results

The results are summarized in tables 1 through 5. Table 1 lists 10 plant species which inhibited tumor growth 70–100 per cent for a 24-hour test period with little effect on embryo growth. The data indicates that in most instances the experimental embryos were larger than the controls. This usually occurs when

TABLE 1

Plant Extracts Inhibiting the Growth of C_3H Mammary Carcinoma in Tumor Bearing Eggs. 24-Hour Test Period.

Family	Plant	USDA Accession No.	No. of Exps.	No. of Eggs	Dosage, mg. of Dried Material	Survival, Control = 100	Avg. Growth for Test Period, Control = 100	
							Tumor	Embryo
Apocynaceae	Strophanthus hispidus	1927-B	2	74	26.3	100	7	109
Capparidaceae	Forchhammeria watsonii	1280	2	65	21.7	78	29	135
Caprifoliaceae	Viburnum macrocephalum	1118	2	87	20.8	100	31	113
Gramineae	Anthoxanthum odoratum	3842	2	80	8.1	97	5	118
Gramineae	Anthoxanthum odoratum	3842	1	44	10.2	90	–16	151
Gramineae	Arundo donax	6063	1	43	21.3	96	0	131
Gramineae	Arundo plinii	1322	3	131	10.0	100	20	127
Gramineae	Arundo plinii	1323	1	42	15.0	78	0	96
Gramineae	Tripsacum laxum	3656	2	81	21.5	99	21	125
Leguminosae	Tamarindus indica	1169	2	69	15.6	115	30	201
Liliaceae	Smilax spinosa	1030	2	82	19.2	92	0	88
Oleaceae	Fraxinus oregona	1264	2	79	12.8	95	20	105

TABLE 2

Plant Extracts Inhibiting the Growth of C_3H Mammary Carcinoma in Tumor Bearing Eggs. 48-Hour Test Period.

Family	Plant	USDA Accession No.	No. of Exps.	No. of Eggs	Temp. of Incubation °F.	Dosage, mg. of Dried Material	Survival, Control = 100	Avg. Growth for Test Period, Control = 100	
								Tumor	Embryo
Apocynaceae	Strophanthus hispidus	1927-B	1	8	99	26.3	75	45	119
Apocynaceae	Strophanthus hispidus	1927-B	1	8	104	26.3	91	19	60
Leguminosae	Peltophorum vogelianum	2223	2	29	99	17.4	78	13	97
Liliaceae	Yucca gloriosa	997	5	40	99	5.3	115	14	90
Liliaceae	Yucca gloriosa	997	2	12	104	5.3	73	–37	84
Liliaceae	Yucca pallida	0–1679	2	13	99	27.8	82	24	121
Liliaceae	Yucca pallida	0–1679	2	13	104	27.8	87	21	103
Liliaceae	Yucca pallida	0–1679	4	32	104	13.9	84	2	105
Moraceae	Maclura pomifera	1164	1	13	99	2.1	77	39	106
Moraceae	Maclura pomifera	1164	1	16	99	6.1	70	4	124
Oleaceae	Chionanthus retusa	1931	4	34	99	25.0	75	44	123
Oleaceae	Chionanthus retusa	1931	3	29	104	25.0	79	–9	104
Pyrolaceae	Chimaphila maculata	1156	1	8	99	8.0	28	–6	103
Pyrolaceae	Chimaphila maculata	1156	2	18	99	4.0	94	28	100
Pyrolaceae	Chimaphila maculata	1156	1	8	104	4.0	100	44	75

TABLE 3

Plant Extracts Inhibiting the Growth of RC Mammary Carcinoma Transplants in dba Mice. One Subdermal Injection. 10-Day Test Period.

Family	Plant	USDA Accession No.	No. of Exps.	No. of Mice	Dosage, mg. of Dried Material	Tumor Growth at Autopsy, Control = 100	Body Weight at Autopsy, Control = 100
Amaryllidaceae	Hymenocallis sp.	1031	3	27	6.6	47	92
Gramineae	Arundo donax	6063	2	18	21.3	37	100
Gramineae	Arundo donax	6062	1	9	23.8	30	97
Oleaceae	Chionanthus retusa	1931	2	18	25.0	56	96

TABLE 4

Plant Extracts with Little or No Effect on C_3H Egg Cultivated Mammary Carcinoma. 24- and 48-Hour Tests

Family	Plant	USDA Accession No.
	Less than 50 per cent tumor growth	
Alismataceae	Sagittaria latifolia var. pubescens	1081
Amaryllidaceae	Hymenocallis sp.	1031
Anacardiaceae	Pistacia vera	1185
Apocynaceae	Apocynaceae	2445
Combretaceae	Terminalia muelleri	1219
Compositae	Trilisa odoratissima	1414
Dilleniaceae	Actinidia chinensis	1179
Ericaceae	Lyonia ligustrina	4194
Euphorbiaceae	Aleurites moluccana	1226
Euphorbiaceae	Antidesma bunius	1176
Gramineae	Bambusa tulda	2895
Gramineae	Cymbopogon nardus	2573
Gramineae	Dendrocalamus strictus	2894
Liliaceae	Yucca arborescens	5923-C
Liliaceae	Yucca filamentosa	0-5381-B
Liliaceae	Yucca freemanii	2570
Liliaceae	Yucca peninsularis	2618
Liliaceae	Yucca schidigera	2777
Liliaceae	Yucca torreyi	6283
Liliaceae	Yucca whipplei	3224
Myricaceae	Myrica rubra	1405
Myrtaceae	Eugenia jambos	2313
Palmae	Styloma pacific	2195
Pinaceae	Abies holophylla	2095
Rosaceae	Rosa sp.	1366
Rutaceae	Citrus amblicarpa	2058
Rutaceae	Phellodendron sp.	1363
Solonaceae	Datura meteloides	1478
	50–60 per cent tumor growth	
Aceraceae	Acer buergerianum	1126
Aquifoliaceae	Ilex vomitoria	1413
Gramineae	Bambusa ventricosa	2896
Liliaceae	Scilla sp.	641
Myrtaceae	Callistemon rigidus	2430
Onagraceae	Fuchsia boliviana	2083
Rosaceae	Aronia arbutifolia	1211
Santalaceae	Pyrularia pubens	1204
Simaroubaceae	Picramnia pentandra	1127
Solonaceae	Solanum carolinense	1150
Umbelliferae	Oxypolis rigidior	1205
	Little or no effect	
Amaryllidaceae	Agave palmeri	1009
Amaryllidaceae	Hymenocallis sonoriensis	1277
Amaryllidaceae	Furcraea sp.	1011
Amaryllidaceae	Furcraea roezlii	1739
Amaryllidaceae	Furcraea gigantea willemetiana	1742
Anacardiaceae	Mangifera indica	1168
Anacardiaceae	Rhus copallina	1143
Apocynaceae	Carissa grandiflora	1218
Asclepiadaceae	Asclepius syriaca	1137
Balsaminaceae	Impatients biflora	1078
Balsaminaceae	Impatiens biflora	1080
Bignoniaceae	Spathodea campanulata	2197
Bromeliaceae	Pitcairnia corallina	2318
Burseraceae	Burseva bipinnata	1093
Cactaceae	Opuntia dillenii	2347
Caprifoliaceae	Abelia grandiflora	1115
Caryophyllaceae	Saponaria sp.	1993
Caryophyllaceae	Saponaria officinalis	1002
Celastraceae	Euonymus phellomana	1361-B
Chemopodiaceae	Chenopodium album	1401
Clethraceae	Clethra alnifolia	1090
Combretaceae	Anogeissus pendula	2361
Commelinaceae	Commelina communis	1160
Compositae	Aster umbellatus	1215
Compositae	Eupatorium perfoliatum	1074

TABLE 4—Continued

Plant Extracts with Little or No Effect on C_3H Egg Cultivated Mammary Carcinoma. 24- and 48-Hour Tests

Family	Plant	USDA Accession No.
Compositae	Eupatorium perfoliatum	1089
Compositae	Helianthus tomentosus	1217
Compositae	Tagetes sp.	1112
Convolvulaceae	Ipomoea pandurata	1162
Cyrillaceae	Cyrilla racemiflora	1114
Ebenaceae	Diospyros ebenaster	1223
Ericaceae	Rhododendron maximum	1213
Euphorbiaceae	Aleurites fordii	1412
Euphorbiaceae	Cnidoscolus urens	1051
Euphorbiaceae	Cnidoscolus tepicensis	1103
Euphorbiaceae	Hura crepitans	2421
Euphorbiaceae	Manihot angustifolia	1048
Fagaceae	Lithocarpus glabra	1406
Flacourtiaceae	Flacourtia montana	2362
Gramineae	Arundo donax	1807
Gramineae	Arundo plinii	6064
Gramineae	Bambusa arundinacea	2893
Gramineae	Bambusa multiplex	1450
Gramineae	Bambusa tuldoides	2897
Lauraceae	Sassafras albidum	1134
Leguminosae	Acacia lutea	2199
Leguminosae	Acacia pycnantha	1121
Leguminosae	Albizzia lebbeck	2314
Leguminosae	Apios americana	1157
Leguminosae	Caesalpinia ferrea	1983
Leguminosae	Cassia fasciculata	1159
Leguminosae	Cassia marginata	2320
Leguminosae	Gleditsia amorphoides	2196
Leguminosae	Haematoxylum campecheanum	2434
Leguminosae	Wisteria floribunda	1116
Liliaceae	Anthericum sp.	1099
Liliaceae	Eucomis sp.	664
Liliaceae	Gasteria sp.	1367
Liliaceae	Lilium superbum	1041
Liliaceae	Milla biflora	1095
Liliaceae	Stenanthium gramineum	1328
Liliaceae	Xerophyllum asphodeloides	1052
Liliaceae	Yucca aloifolia	2793
Liliaceae	Yucca arizonica	2512
Liliaceae	Yucca arborescens	0-5819
Liliaceae	Yucca arborescens	0-5823
Liliaceae	Yucca arborescens	0-5852-A
Liliaceae	Yucca arborescens	0-5923-D
Liliaceae	Yucca baccata	0-5847-A
Liliaceae	Yucca baccata	0-5848
Liliaceae	Yucca carnerosana	2381
Liliaceae	Yucca filamentosa	0-5381-D
Liliaceae	Yucca gloriosa	0-2508
Liliaceae	Yucca peninsularis	2595
Liliaceae	Yucca peninsularis	2600
Liliaceae	Yucca schidigera	2601
Liliaceae	Yucca torreyi	5934
Liliaceae	Yucca valida	1734
Lythraceae	Woodfordia fruticosa	2358
Malpighiaceae	Hiptage benghalensis	2365
Malpighiaceae	Stigmaphyllon fulgens	2366
Martyniaceae	Martynia arenaria	1288
Moraceae	Coussapoa sp.	1964
Musaceae	Strelitzia alba	2407
Myrtaceae	Eugenia dalbergioides	2406
Myrtaceae	Eugenia supra-axillaris	2364
Myrtaceae	Myrciaria cauliflora	1167
Myrtaceae	Psidium guineense	1173
Myrtaceae	Syzygium lineatum	2193
Oleaceae	Fraxinus chinensis	1364-A
Oleaceae	Fraxinus chinensis	1364-B
Oleaceae	Ligustrum sp.	1365-A
Palmae	Chamaedorea erumpens	2346
Palmae	Heterospathe elata	2311

TABLE 4—Continued

Plant Extracts with Little or No Effect on C_3H Egg Cultivated Mammary Carcinoma. 24- and 48-Hour Tests

Family	Plant	USDA Accession No.
Phytolaccaceae	Phytolacca americana	1152
Phytolaccaceae	Phytolacca americana	1153
Pinaceae	Callitris quadrivalvis	2403
Proteaceae	Macadamia ternifolia	2316
Rosaceae	Cotoneaster foveolata	1362-A
Rosaceae	Cotoneaster foveolata	1362-B
Rosaceae	Spiraea alba	1207
Rosaceae	Spiraea bumalda Anth. Waterer	1120
Rubiaceae	Psychotria bacteriophila	2345
Rubiaceae	Randia formosa	2360
Rutaceae	Zanthoxylum bungei	1353
Salicaceae	Populus berolinensis	1067
Sapindaceae	Litchi chinensis	1129
Sapotaceae	Lucuma salicifolia	1128
Saxifragaceae	Philadelphus henryi	1360-A
Simaroubaceae	Picrasma quassioides	1355
Solanaceae	Brunfelsia americana	2404
Symplocaceae	Symplocos paniculata	1350
Symplocaceae	Symplocos paniculata	1351
Tamaricaceae	Tamarix juniperina	1988
Taxaceae	Cephalataxus sp.	2094-A
Theaceae	Franklinia alatamaha	1055
Tiliaceae	Berrya ammonilla	2025
Tiliaceae	Carpodiptera ameliae	1175
Tiliaceae	Grewia biloba	1359-A
Tiliaceae	Grewia biloba	1359-B
Tiliaceae	Tilia dasystyla	1069
Tiliaceae	Triumfetta polyandra	1094
Typhaceae	Typha angustifolia	1087
Verbenaceae	Congea tomentosa	2194
Vitaceae	Ampelopsis sp.	1101
Vitaceae	Ampelopsis quinquefolium	1141

TABLE 5

Plant Extracts with Little or No Effect on the Growth of dba Mouse Mammary Carcinoma Transplants. 10-Day Test Period

Family	Plant	USDA Accession No.
	Less than 60 per cent tumor growth	
Amaryllidaceae	Beschornera yuccoides	1715
Compositae	Tithonia diversifolia	2044
Ebenaceae	Diospyros montana	1224
Ericaceae	Leucothoe sp.	1986
Euphorbiaceae	Euphorbia corollata	1131
Juglandaceae	Pterocarya stenoptera	1404
Leguminosae	Derris pterocarpus	2032
Liliaceae	Liriope graminifolia	1060
Liliaceae	Yucca arborescens	5861
Liliaceae	Yucca filamentosa	0-5381-B
Liliaceae	Yucca reverehonii	0-1677
Liliaceae	Yucca whipplei	3224
Malvaceae	Hisbicus tricuspis	2224
Moraceae	Maclura pomifera	1164
Myrtaceae	Eugenia jambos	2313
Myrtaceae	Psidium guajava	1172
Pinaceae	Juniperus barbadensis	2433
Pinaceae	Larix sp.	1987
Rutaceae	Citrus amblycarpa	2058
Sapindaceae	Blighia sapida	1171
Sparganiaceae	Sparganium americanum	1214

TABLE 5—Continued

Plant Extracts with Little or No Effect on the Growth of dba Mouse Mammary Carcinoma Transplants. 10-Day Test Period

Family	Plant	USDA Accession No.
	60–80 per cent tumor growth	
Amaryllidaceae	Hymenocallis sonoriensis	1277
Amaryllidaceae	Furcraea roezlii	1739
Apocynaceae	Carissa grandiflora	1218
Compositae	Helianthus tomentosus	1217
Ericaceae	Kalmia latifolia	1212
Ericaceae	Rhododendron maximum	1213
Euphorbiaceae	Cnidoscolus tepicensis	1103
Gramineae	Arundo plinii	6064
Leguminosae	Acacia pycnantha	1121
Leguminosae	Caesalpinia ferrea	1983
Leguminosae	Cassia marginata	2320
Leguminosae	Wisteria floribunda	1116
Liliaceae	Eucomis sp.	664
Liliaceae	Scilla sp.	641
Liliaceae	Yucca arizonica	2512
Liliaceae	Yucca peninsularis	2600
Rosaceae	Cotoneaster foveolata	1362-A
Rosaceae	Spiraea bumalda Anth. Waterer	1120
Rubiaceae	Psychotria bacteriophila	2345
Saxifragaceae	Hydrangea petiolaris	1110
Tiliaceae	Grewia biloba	1359-A
	Little or no effect on tumor growth	
Aceraceae	Acer buergerianum	1126
Amaryllidaceae	Agavo palmeri	1009
Amaryllidaceae	Furcraea sp.	1011
Amaryllidaceae	Furcraea gigantea willemetiana	1742
Anacardiaceae	Mangifera indica	1168
Anacardiaceae	Rhus copallina	1143
Asclepiadaceae	Asclepius syriaca	1137
Aquifoliaceae	Ilex vomitoria	1413
Balsaminaceae	Impatiens biflora	1078
Balsaminaceae	Impatiens biflora	1080
Bignoniaceae	Spathodea campanulata	2197
Bromeliaceae	Pitcairnia corallina	2318
Burseraceae	Bursera bipinnata	1093
Cactaceae	Opuntia dillenii	2347
Caprifoliaceae	Abelia grandiflora	1115
Caryophyllaceae	Saponaria sp.	1993
Caryophyllaceae	Saponaria officinalis	1002
Celastraceae	Euonymus phellomana	1361-B
Chenopodiaceae	Chenopodium album	1401
Clethraceae	Clethra alnifolia	1090
Combretaceae	Anogeissus pendula	2361
Commelinaceae	Commelina communis	1160
Compositae	Aster umbellatus	1215
Compositae	Eupatorium perfoliatum	1074
Compositae	Eupatorium perfoliatum	1089
Compositae	Tagetes sp.	1112
Convolvulaceae	Ipomoea pandurata	1162
Cyrillaceae	Cyrilla racemiflora	1114
Ebenaceae	Diospyros ebenaster	1223
Euphorbiaceae	Aleurites fordii	1412
Euphorbiaceae	Cnidoscolus urens	1051
Euphorbiaceae	Hura crepitans	2421
Euphorbiaceae	Manihot angustifolia	1048
Fagaceae	Lithocarpus glabra	1406
Flacourtiaceae	Flacourtia montana	2362
Gramineae	Arundo donax	1807
Gramineae	Bambusa arundinacea	2893
Gramineae	Bambusa multiplex	1450
Gramineae	Bambusa ventricosa	2896
Gramineae	Bambusa tuldoides	2897
Lauraceae	Sassafras albidum	1134
Leguminosae	Acacia lutea	2199
Leguminosae	Albizzia lebbeck	2314
Leguminosae	Apios americana	1157

TABLE 5—Continued

Plant Extracts with Little or No Effect on the Growth of dba Mouse Mammary Carcinoma Transplants. 10-Day Test Period

Family	Plant	USDA Accession No.
Leguminosae	Cassia fasciculata	1159
Leguminosae	Gleditsia amorphoides	2196
Leguminosae	Haematoxylum campecheanum	2434
Liliaceae	Anthericum sp.	1099
Liliaceae	Gasteria sp.	1367
Liliaceae	Lilium superbum	1041
Liliaceae	Milla biflora	1095
Liliaceae	Stenanthium gramineum	1328
Liliaceae	Xerophyllum asphodeloides	1052
Liliaceae	Yucca aloifolia	2793
Liliaceae	Yucca arborescens	0-5819
Liliaceae	Yucca arborescens	0-5823
Liliaceae	Yucca arborescens	0-5852-A
Liliaceae	Yucca arborescens	0-5923-D
Liliaceae	Yucca baccata	0-5847-A
Liliaceae	Yucca baccata	0-5848
Liliaceae	Yucca carnerosana	2381
Liliaceae	Yucca filamentosa	0-5381-D
Liliaceae	Yucca gloriosa	0-2508
Liliaceae	Yucca pallida	0-1679
Liliaceae	Yucca peninsularis	2595
Liliaceae	Yucca schidigera	2601
Liliaceae	Yucca torreyi	5934
Liliaceae	Yucca valida	1734
Lythraceae	Woodfordia fruticosa	2358
Malpighiaceae	Hiptage benghalensis	2365
Malpighiaceae	Stigmaphyllon fulgens	2366
Martyniaceae	Martynia arenaria	1288
Moraceae	Coussapoa sp.	1964
Musaceae	Strelitzia alba	2407
Myrtaceae	Callistemon rigidus	2430
Myrtaceae	Eugenia dalbergioides	2406
Myrtaceae	Eugenia supra-axillaris	2364
Myrtaceae	Myrciaria cauliflora	1167
Myrtaceae	Psidium guineese	1173
Myrtaceae	Syzygium lineatum	2193
Oleaceae	Fraxinus chinensis	1364-A
Oleaceae	Fraxinus chinensis	1364-B
Oleaceae	Ligustrum sp.	1365-A
Onagraceae	Fuchsia boliviana	2083
Palmae	Chamaedorea erumpens	2346
Palmae	Heterospathe elata	2311
Phytolaccaceae	Phytolacca americana	1152
Phytolaccaceae	Phytolacca americana	1153
Pinaceae	Callitris quadrivalvis	2403
Proteaceae	Macadamia ternifolia	2316
Rosaceae	Aronia arbutifolia	1211
Rosaceae	Cotoneaster foveolata	1362-B
Rosaceae	Spirea alba	1207
Rubiaceae	Randia formosa	2360
Rutaceae	Zanthoxylum bungei	1353
Salicaceae	Populus berolinensis	1067
Santalaceae	Pyrularia pubens	1204
Sapindaceae	Litchi chinensis	1129
Sapotaceae	Achras sapota	1166
Sapotaceae	Lucuma salicifolia	1128
Saxifragaceae	Philadelphus henryi	1360-A
Simaroubaceae	Picrasma quassioides	1355
Simaroubaceae	Picramnia pentandra	1127
Solanaceae	Brunfelsia americana	2404
Solanaceae	Solanum carolinense	1150
Symplocaceae	Symplocos paniculata	1350
Symplocaceae	Symplocos paniculata	1351
Tamaricaceae	Tamarix juniperina	1988
Taxaceae	Cephalataxus sp.	2094-A
Theaceae	Franklinia alatamaha	1055
Tiliaceae	Berrya ammonilla	2025
Tiliaceae	Carpodiptera ameliae	1175
Tiliaceae	Grewia biloba	1359-B

TABLE 5—Continued

Plant Extracts with Little or No Effect on the Growth of dba Mouse Mammary Carcinoma Transplants. 10-Day Test Period

Family	Plant	USDA Accession No.
Tiliaceae	Tilia daasystyla	1069
Tiliaceae	Triumfetta polyandra	1094
Typhaceae	Typha angustifolia	1087
Umbelliferae	Oxypolis rigidior	1205
Verbenaceae	Congea tomentosa	2194
Vitaceae	Ampelopsis sp.	1101
Vitaceae	Ampelopsis quinquefolium	1141

the egg tumors are reduced in size. The family, *Graminae*, contained 4 active species in 3 genera.

These extracts appeared to be well tolerated by the chick embryo. A one-day test does not, however, reveal the full toxic possibilities an extract may have since a longer period of time may be necessary to reveal the full effect. Usually the embryo weight changes give a fair indication of the reaction of nontumor tissue to a particular extract. Table 2 lists 7 plant extracts which, on the basis of a 48-hour test period inhibited tumor growth 55–100 per cent as compared to the growth in the controls. Slightly more than 10 per cent of the plant species tested gave extracts which, by the egg cultivated method, inhibited tumor growth 55–100 per cent.

The RC tumor transplants in mice have proved to be a more difficult test for the plant extracts. As table 3 reveals, extracts from 3 species inhibited tumor growth 44–70 per cent. This represented less than 2 per cent of the number of species tested.

Tables 4 and 5 record the results with extracts having a lesser or negligible effect on tumor growth. It seems important to include these more or less negative data for the benefit of other investigators using this approach to cancer chemotherapy.

Discussion

Reports of research in cancer chemotherapy are mainly concerned with data which may indicate hopeful approaches to the problem of cancer control. None of the plant extracts processed to date have, as yet, given results which promise practical application to cancer therapy. Some of the extracts do merit, and will be given, further attention.

The chief importance of the data presented is that it indicates the anticancer possibilities of extracts of the naturally occurring compounds present in members of the plant kingdom and so lends encouragement to further work in this field.

Summary

Extracts from 161 plant species were tested for their effect on the growth of egg-yolk-sac-cultivated mouse mammary tumors. Seventeen plant species inhibited tumor growth 55–100 per cent as compared with the saline-treated controls. The family, *Graminae*, has given effective extracts from 4 species which included 3 genera.

Three plant species out of a series of 153 species gave extracts which inhibited transplants of the RC tumor in mice 44–70 per cent.

The number of plant extracts with anticancer activity in relation to the total number tested indicates that the use of these naturally occurring compounds is a promising approach to a cancer chemotherapy.

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STUDY OF MITOSIS IN THE COMPENSATORY HYPERTROPHIC KIDNEY FOLLOWING UNILATERAL NEPHRECTOMY IN THE RAT[a]

KAZUO OGAWA,[b] AND JOHN G. SINCLAIR

The investigations of Arataki (1), Saphir (3), and others (2, 5), indicate that in the compensatory hypertrophic kidney, the glomeruli become enlarged, due primarily to cellular hypertrophy, whereas in the tubules and collecting ducts both cellular hypertrophy and hyperplasia occur, as evidenced by an increased mitotic activity.

Rollanson (2) found that the mitotic activity in the cortex, outer medulla and inner medulla of the rat was largely restricted to the 2nd day after the unilateral nephrectomy, but Sulkin found the peak of mitotic activity in 72 to 240 hours after the operation (5).

Because of this discrepancy it was decided to reinvestigate the time period of maximum mitotic activity in the cortex, outer medulla and inner medulla of the compensatory hypertrophic kidney following unilateral nephrectomy.

Materials and Methods

Holtzman strain male albino rats, weighing 150 to 170 gm., were used. The rats were unilaterally nephrectomized and kept in the Animal Hospital of The University of Texas Medical Branch. They were sacrificed on the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th, 10th and 23rd postoperative days respectively by a blow on the neck. Mitotic counts were made on one rat of each of these stages. The kidneys were removed and median transverse blocks were fixed in 10 per cent neutral formalin solution. Following the ordinary paraffin technique, serial sections were cut at 6 μ. and stained with the usual hematoxylin-eosin and iron-hematoxylin-eosin.

The number of mitoses per 200 fields under magnification of 400x was determined in each of the three zones of each kidney. The kidneys removed at the time of unilateral nephrectomy served as controls.

TABLE 1

Mitotic Activity in the Compensatory Hypertrophic Kidney

Number of mitotic cells per 200 fields (400x) in three zones.

	Normal	1	2	3	4	5	6	7	8	9	10	23
		days following unilateral nephrectomy										
Cortex	1	0	31	15	15	17	9	22	12	6	8	2
Outer Medulla	0	1	25	19	21	21	10	36	21	5	10	0
Inner Medulla	0	0	8	3	8	10	5	10	7	2	1	0
Total	1	1	65	37	44	48	24	68	40	13	19	2

Results

The results are shown in table 1 and figures I and II. In the cortex, particularly tubular cells, there occurred two peaks of mitotic activity, the higher on the 2nd postoperative day (31 dividing cells per 200 fields) and the lower on the 7th postoperative day (22 mitotic cells per 200 fields). Occasionally mitotic figures were found in the Bowman's capsule, but no dividing cells were seen in the cells of glomeruli.

The outer medulla showed a reversed pattern. The peak of

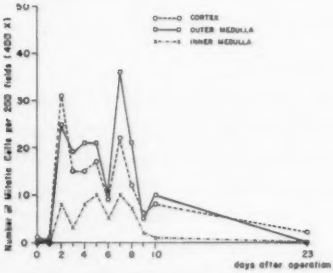

FIG. I. Mitotic activity in the compensatory hypertrophic kidney. Cortex shows the highest mitotic activity on the 2nd postoperative day, whereas outer medulla shows the maximum mitotic activity on the 7th postoperative day. Inner medulla showed considerably lower activity, and no particular peak.

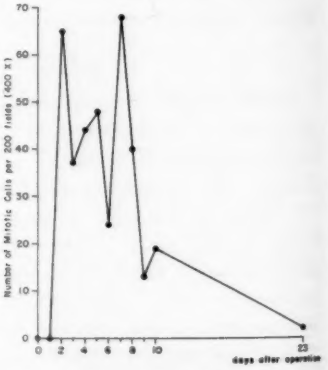

FIG. II. Mitotic activity in the compensatory hypertrophic kidney. (Kidney as a whole.) Bimodality is shown in this figure. The first peak appears on the 2nd postoperative day, and the second peak on the 7th postoperative day.

mitotic activity was shown on the 2nd (25 cells in mitosis) and 7th (36 mitosis) postoperative days. The inner medulla showed no particular peak, and the mitotic activity was considerably lower than that obtained in the cortex and outer medulla. The majority of the mitotic cells were found in the collecting ducts.

On the 2nd day, the greatest absolute increase of mitotic activity was shown in the cortex, but later the outer medulla showed higher mitotic activity than the cortex, particularly on the 7th postoperative day.

Taking kidney as a whole, two prominent peaks of mitotic activity occur on the 2nd and 7th days (Fig. II). The cells in three zones respond to unilateral nephrectomy with respect to mitosis within 2 days following operation and the mitotic activity in three zones decreased significantly in 10 days and returned to normal in 23 days.

Discussion

As is apparent from figures I and II, there are two peaks (bimodality) in the mitotic activity in the compensatory hypertrophic kidney; on the 2nd and 7th postoperative days. The first peak is in agreement with work of Rollanson (2). The second peak appears to be rather comparable to the one described by Sulkin (5) in the compensatory hypertrophic kidney and to those of Sinclair (4) during the healing process following experimentally produced myocardial infarction in the dog. Further, interestingly enough, though the cortex showed the highest activity in the first peak, the outer medulla showed maximum mitotic activity in the second peak. The inner medulla showed the smallest number of mitotic figures throughout the investigated stages.

In the regenerating mouse liver after partial hepatectomy, Wilson *et al.* (6) found the highest mitotic activity on the 3rd day and by the 7th day the mitotic activity dropped nearly to normal, but subsequently showed another small peak on the 8th postoperative day.

In the liver, it is not uncommon that the initial phase of recovery from partial hepatectomy is followed by a period of several weeks in which variable amounts of focal necrosis are observed, and this necrosis is severe enough to produce secondary regenerative activity. The second peak seen on the 8th day might be due to the presence of this focal necrosis. In the kidney, how-

ever, focal necrosis following initial recovery from unilateral nephrectomy is not found and therefore the second peak, seen on the 7th day, could not possibly be due to a focal necrosis.

As already mentioned, the cells in three zones showed almost immediate repsonse to unilateral nephrectomy, above all the cortex showed the highest mitotic activity on the 2nd day. Later, on the 7th day, the outer medulla showed the highest mitotic activity. The first peak represents primarily the increased mitotic activity of the cortex and the second peak represents the increased mitotic activity of the outer medulla. This might indicate a difference in response to unilateral nephrectomy between the cortex and the outer medulla, and this difference in response might be the cause of the bimodality of mitotic activity observed in the compensatory hypertrophic kidney.

Summary

1. The mitotic activity in the cortex, outer medulla and inner medulla of the compensatory hypertrophic kidney following unilateral nephrectomy was studied.

2. The three zones showed almost immediate response to unilateral nephrectomy. The mitotic activity in two zones increased considerably, within 2 days following operation, primarily in the cortex. A second increase appeared on the 7th day, particularly in the outer medulla. The possibility is postulated that this difference in response may be due to existing differences in the reactivity of the cells of cortex and those of the outer medulla.

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EFFECTS OF LACTATION ON THE NEUROHYPOPHYSIS OF THE RAT[a]

EDWARD G. RENNELS[b]

It is now well known that the posterior pituitary hormones are closely associated in some manner with the Gomori-positive neurosecretory material of the neurohypophysis. Whether the latter material is in the nature of a carrier substance for the water soluble hormones or is more intimately related to them is not yet clear, however. It has been claimed recently that the hormones are a part of the neurosecretory material (Sloper, 1955) although earlier evidence for their being different chemical substances seemed acceptable (Hild and Zetler, 1953). The regulatory mechchanism for altering posterior pituitary function has received a great deal of attention. It now seems clear that qualitatively different kinds of stimuli are effective in bringing about a discharge of the hormones. Thus, changes in osmotic pressure of the blood or the nervous stimuli produced by suckling young are capable of inducing posterior lobe hormonal discharge. Since the work of Du Vigneaud and his co-workers (Du Vigneaud *et al.*, 1953 and 1953a) in defining the chemical nature of vasopressin and oxytocin, it is generally held that these two polypeptides represent the hormones, with the antidiuretic activity residing in the vasopressin molecule. It is thoroughly possible, however, that a single large molecule having these two polypeptides as side chains may be the physiological hormone that is released into the circulation. Evidence for considerable disparity between the release of vasopressin and oxytocin under the influence of different stimuli may be presented as favoring the separate nature and secretion of these two active principles (Harris, 1955). On the other hand, there is equally good evidence for the simultaneous release of vasopressin and oxytocin under a variety of physiological stimuli.

Studies of neurosecretion and the associated hormones during the course of lactation have been rather few in number (See Cowie and Falley, 1957, for review). The need for oxytocin in the milk "letdown" phenomenon has been demonstrated by a number of workers and its release is dependent on the nervous stimulation of suckling. It has been claimed that lactating animals

contain a greatly reduced amount of oxytocin in their posterior pituitary glands, whereas the vasopressin level is not significantly changed (Dicker and Tyler, 1953). In view of these findings our own preliminary results were interpreted as indicative of a closer association between vasopressin and neurosecretion than between oxytocin and neurosecretion (Rennels *et al.*, 1955). While the major emphasis of the present study was directed toward investigating the changes in the neurohypophysis in lactation, an attempt was also made to do a correlative study of the pituitary content of vasopressin, oxytocin and neurosecretion under a variety of experimental conditions known to alter posterior lobe physiology. In this manner it was hoped to gain further insight into the nature of the material secreted into the blood and its relation to stainable neurosecretion. On the basis of the data to be presented it seems necessary to conclude that the two hormones are liberated simultaneously no matter what the initiating stimulus and further that there is always a very close relationship between the behavior of both hormones and stainable neurosecretion.

Materials and Methods

Animals. The rats used in this study were either obtained directly from the Holtzman Rat Company or were animals raised from this stock in our animal colony. The animals were kept in air conditioned rooms and maintained on Purina Laboratory Chow.

Male animals were used in our initial studies in which attempts were made to increase or decrease the pitutitary stores of neurosecretion and hormones. The groups of male rats used for these studies are indicated in table 1 and the results obtained are given in figures I and II. This part of the study will be referred to as Experiment 1.

The adrenalectomized animals were maintained on 1 per cent NaCl for the 7–10 day period before they were killed. The dehydrated rats were given

TABLE 1

Group	Number of animals		
	For neurosecretion studies	For hormone assay	
Normal controls	10	(8)	35
Adrenalectomized (7–10 days)	5	(6)	27
Dehydrated (2.5 per cent NaCl)	5	(3)	18
Alloxan-treated	5	(2)	8
Cortisone-treated	5	(2)	9
ACTH-treated	5	(3)	13

a 2.5 per cent NaCl solution to drink for a 10–12 day period. This procedure was found to produce a severe state of dehydration and the animals were all in poor condition at the time they were killed. The average body weight of the rats in these two groups was approximately 150 grams. Control animals of about the same weight were used for determining control levels of hormones and neurosecretion in the posterior pituitary. The numbers in parentheses in table 1 refer to the groups of animals that were used in preparing separate extracts for hormone assay.

Alloxan diabetes was produced by giving male rats weighing about 220 grams a single intravenous injection of 10 mgms. of alloxan. In some animals it was necessary to give a second injection in order to produce diabetic symptoms. All of the rats used in this experiment were drinking in excess of 150 ml. of water daily at the time they were killed and exhibited a marked glucosuria. They were all killed 20 days after the initial injection of alloxan.

Cortisone (1 mgm./day) and adrenocorticotrophic hormone (4 mgm./day) was administered by subcutaneous injection to separate groups of male rats weighing about 220 grams for a 20 day period. These treatments were used because of the known effects of adrenal cortical hormones on water and electrolyte metabolism.

The female rats used for pregnancy and lactation studies were obtained from our own colony and records were available as to the number of litters each had borne. In general, the litter size was kept at 6 young. In a few experiments, however, the number of young was increased and foster litters were supplied at the end of a lactation period. Four females were encountered which delivered normal young but which failed to lactate and these have been placed in a separate category for neurosecretion study. (Lactation failure group, table 2). For control purposes, neurosecretion has also been studied in a group of old males, and in a group of old nulliparous females. The groups of animals studied in connection with lactation are indicated in table 2. This study will be referred to later as Experiment 2 and the results are shown graphically in figure III.

B. Methods of Analysis

Neurosecretion. Neurosecretion has been studied by the use of 5 μ paraffin sections of the pituitary glands, stained with either Gomori's chrome alum

TABLE 2

Group	Number of Animals	
	For neurosecretion studies	For hormone assay
Non-lactating controls	39	18
Lactating (4th litter)	94	21
Lactation Failure (4th litter)	4	
Pregnant	7	
Old nulliparous	4	
Old males	6	

hematoxylin (CAH) or an aldehyde fuchsin (AF) method. Both methods give a selective coloration of the neurosecretory material of the posterior pituitary but aldehyde fuchsin gives a somewhat more intense coloration. A number of different fixatives were tried but the most satisfactory preservation of neurosecretion was obtained by 6–8 hours fixation in Zenker-formol. In our early work on this problem we routinely studied serially sectioned glands stained with either CAH or AF. It soon became apparent that minor differences in the content of neurosecretion among a group of animals could not be measured reliably by such a method. We have since resorted to a method of section mounting which enables one to obtain uniform staining of sections from a number of glands on a single slide. Details of the mounting and staining procedures have been published (Rennels, 1957). Furthermore, adjacent sections can, by this method, be placed on different slides and subjected to different staining procedures. By this mounting procedure, comparable sections at every 0.1 mm. level of all glands in a given experiment were placed on separate slides and stained with CAH. Other slides containing adjacent sections were then stained with AF. In evaluating the amount of neurosecretion, three workers[c] who were familiar with the general problem examined the slides and rated each section on a scale from 0–5 according to the amount and staining intensity of the neurosecretory material (Plate I). A system of coding was used in preparing the slides such that the three examiners did not have knowledge of the groups to which the various glands belonged. From 5–9 sections from each gland were evaluated following CAH and AF staining. An average value was obtained for the ratings given each gland and from these mean values for each group of animals.

Oxytocin. Pooled glands representing each group of animals were ground in a mortar and extracted with 3 ml. of boiling acetic acid (0.25 per cent). This suspension was filtered and the filtrate kept frozen until such time as it was assayed for hormone content. The details of this method and the method used for determining the pituitary content of oxytocin and vasopressin have been recently published (Rennels and Russell, 1957). A modification of Coon's rooster method (Coon, 1939) was used in determining the oxytocin content of the pituitary glands. The number of separate extracts assayed for each experimental group of Experiment 1 is given in table 1. Fifty-two roosters were used in performing these assays.

Vasopressin. Each extract was also analyzed for its content of vasopressin by a rat pressor assay method similar to that described by Landgrebe, Macauley and Waring (1946). All hormonal values were computed in terms of milliunits per pituitary gland. A total of 196 male rats were used in making pressor determinations.

Results

Role of Pituicytes. We have described an intimate relationship between the nerve terminals containing neurosecretory material and osmiophilic glandular pituicytes (Rennels and Drager, 1955). It was suggested on the basis of this relationship that these pituicytes may play a role in the release of the hormones into the

PLATE I. These figures represent sections of male pituitary gland is stained with aldehyde fuchsin for neurosecretion. The animal groups represented and the values recorded in each case were: figure 1 dehydration (O), figure 2 dehydration (1), figure 3 alloxan treatment (2), figure 4 ACTH treatment (3), figure 5 normal control (4), figure 6 ACTH treatment (5).

blood vessels. Hypertrophic pituicytes were found to be a striking feature of the posterior lobe of multiparous females. These observations have been extended and confirmed so that it can now be stated that, in general, these pituicytes are larger and more prominent in multiparous, lactating females than in any other group of animals that have been studied. This fact indicates that these cells may play some special role in the process of oxytocin release. A separate experiment was performed to see if these cells could be modified by increasing the functional demands on the neurohypophysis as regards vasopressor hormone secretion. A group of 6 rats were alternately dehydrated by water deprivation for periods of 7 days and rehydrated by giving water for periods of 3 days. This schedule was repeated through 11 water deprivation periods extending over 3½ months. At the end of this time the animals weighed no more than 120 grams as compared to more than 300 grams for their untreated controls. Some of these rats were killed in the dehydrated phase and others after having been given free access to water for the 3 day period. It was thought that this repetitive high demand on posterior lobe function would perhaps modify the pituicytes. It was found that these cells were not noticeably modified although the content of neurosecretion was quite different in the different animals depending on when they were killed. Those killed at the end of a water deprivation period showed very little neurosecretory material whereas the others contained a considerable amount.

Relationship of Neurosecretion to Oxytocin and Vasopressin. It was our early opinion that there was no marked alteration of neurosecretion during either parturition or lactation (Drager and Rennels, 1955). In view of the rather wide degree of individual variation in the amount of such material present and the several technical variables involved in evaluating it, we decided to study this problem more carefully by the technique described. This method of quantitating the amount of stainable material was then applied to the glands of 34 animals in Experiment 1 and 52 animals in Experiment 2. In evaluating the amount of neurosecretion only full unit steps on the 0-5 scale were used. Sections typical of these gradations in neurosecretory content are illustrated in plate I. (In all the cases illustrated there was full agreement in the rating of the neurosecretion by the three examiners.) The results are given in graphic form in figures I and II for Ex-

periment 1 and in figure III for Experiment 2. Assay data for the oxytocin and vasopressin content of glands representing the same experimental groups are also given in these figures. The ordinate scale of these graphs reads in milliunits of oxytocin or vasopressin per gland. Neurosecretion levels in arbitrary units (scale = 0-5) is represented by these values divided by 100. For the hormone assays the number of determinations and the standard error of the mean is indicated by the number and horizontal line above each column. The numbers above the neurosecretion columns refer to the number of glands studied and the horizontal line indicates the standard error of the mean of the values obtained in arbitrary units of neurosecretion. Differences between group means have been analyzed statistically.

A careful scrutiny of the data presented in figures I and II which deal with the results obtained with male animals of Experiment 1 reveals the following:

1. It appears that only severe dehydration can effectively discharge all three components (oxytocin, vasopressin, and neurosecretion) to a very low level.

2. Diabetes mellitus, as produced by alloxan, was the next most effective procedure for discharging these materials.

3. Adrenalectomy led to an increase in oxytocin content and in the amount of neurosecretion but did not alter the level of vasopressin.

4. The content of both hormones was increased somewhat by cortisone or ACTH treatment but the amount of neurosecretion was not appreciably altered.

5. The amount of neurosecretion present in two control groups of male rats (Figs. I and II) is significantly different. (These control groups were different in body weight and this was the basis for separating the data of figures I and II.) The assay data for oxytocin and vasopressin, however, gave no indication of a similar difference.

In connection with the studies concerned with lactation (Experiment 2) the data of figure III reveal the following points:

1. The content of both hormones is significantly greater in the pituitary glands of female rats than in those of males.

2. The content of both hormones declines during lactation with some recovery seen near the end of the lactation period. The maximal drop is seen at 14–15 days lactation when the level of

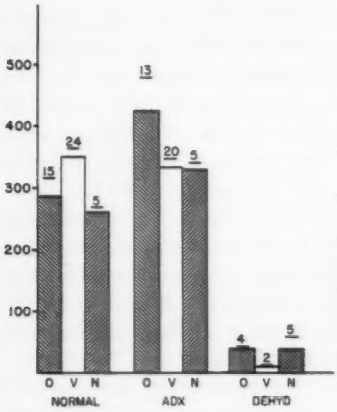

FIG. I. Effect of adrenalectomy (ADX), and dehydration (DEHYD) on the pituitary content of oxytocin (O), vasopressin (V) and neurosecretion (N). The number of determinations and the standard error of the mean are indicated by the number and horizontal line above each column. See text for units of measurement.

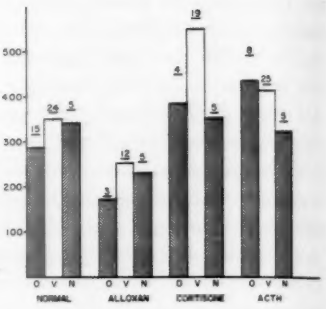

FIG. II. Effect of alloxan diabetes, cortisone treatment and ACTH treatment on the pituitary content of oxytocin (O), vasopressin (V) and neurosecretion (N). The number of determinations and the standard error of the mean are indicated by the number and horizontal line above each column. See text for units of measurement.

oxytocin is only 41 per cent of the control level and that of vasopressin 53 per cent of the control level.

3. Similar but relatively smaller changes in the amount of neurosecretion occur in association with lactation. In this case the minimum level is seen at 18–22 days lactation when 69 per cent of the control level is present. If either the non-pregnant, non-lactating female which has borne 4 litters (N_1) or the pregnant female (N_2) is taken as the control, there is a significant decrease in neurosecretion during lactation.

4. The level of neurosecretion is higher in pregnant females (N_2) or in normal non-pregnant, non-lactating females (N_1) than in old males (N_3), old nulliparous females (N_4) or in females which have lost their litter due to a failure to lactate (N_5).

Discussion

The finding of hypertrophied pituicytes associated with the phenomenon of lactation seems to warrant further study. It may

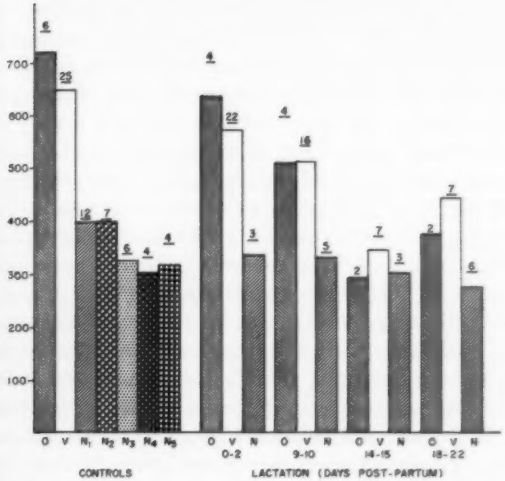

FIG. III. Pituitary content of oxytocin (O), vasopressin (V) and neurosecretion (N) in lactating and non-lactating rats. Control glands for hormone assays were obtained from non-lactating females which had previously borne four litters. Five groups of controls for lactation are indicated for neurosecretion, N_1 = non-lactating females which had previously borne four litters, N_2 = pregnant females, N_3 = old males, N_4 = old nulliparous females. N =females which failed to nurse fourth litter. All lactating rats were killed while nursing fourth litter. See text for units of measurement.

be that these cells are concerned with the release of oxytocin or it may equally well be that their hypertrophy is secondary to some of the many hormonal changes which accompany pregnancy, parturition or lactation. It seems doubtful if their hypertrophy is a simple reflection of hyperactivity of the neurohypophysis in view of the failure of a series of hormonal depletions by dehydration to alter their morphology. It is, of course, also possible that different mechanisms operate in the release of oxytocin and vasopressin. A few attempts were made to prolong the lactation period by substitution of foster litters at the end of a normal lactation period. This procedure did not appreciably alter the appearance of pituicytes nor did it affect the neurosecretion picture beyond the alteration seen in normal lactation. It must be concluded, therefore, that the significance of hypertrophic pituicytes in the posterior lobe of lactating rats is still obscure.

When one considers the experimental errors inherent in the

extractive and assay procedures the most striking feature of the results obtained in this study is the close approximation to unity in the oxytocin: vasopressin ratios seen in the various experimental groups. This is in agreement with most of the assay data for the rat found in the literature (see Harris, 1955 for review). This fact is potent evidence favoring the view that the posterior lobe liberates oxytocin and vasopressin simultaneously and in equivalent amounts in response to a variety of stimuli. Considering all the groups studied, the only case where there appears to be a notable exception to this generalization is in the cortisone-treated group in which the vasopressin content is significantly increased without much change in oxytocin or neurosecretion. It should be noted in this case that the oxytocin value is based on only 4 determinations and therefore its accuracy is somewhat uncertain. The variability of the assay values for oxytocin was, in general, a good deal higher than those of vasopressin. Unfortunately, the rooster method is rather insensitive and the number of assays was therefore somewhat limited in several cases due to the limited quantity of extract.

Both cortisone and ACTH tended to increase the pituitary stores of oxytocin and vasopressin without producing much apparent effect on the level of neurosecretion. This may be explained in part by the nature of our method of evaluating neurosecretion. Since we established a rating of 5 as representing maximal storage of neurosecretion it can readily be seen that with a normal level of 3.4, one is more limited in measuring an increase than in measuring a decrease in neurosecretion. This deficiency in the method is even more important in interpreting the results shown in figure III. The studies carried out on male animals represented in figures I and II (Experiment 1) were done primarily to establish a suitable method for evaluating neurosecretion in a quantitative manner. When Experiment 2 was completed and the slides were evaluated it became evident that we were somewhat limited by having established 5 as a maximal value. In other words, the value of 4.0 shown in figure III as representative of nonlactating (N_1) and pregnant (N_2) females should probably be somewhat higher relative to the value of 3.4 which represents the average level in normal male glands (Fig. II). If we had reversed the order in which the slides of the two experiments were evaluated a somewhat more accurate reflection

of the difference in neurosecretion might have been obtained. Nevertheless, it is felt that the values in arbitrary units obtained for neurosecretion by this technique are probably subject to no greater error than are the bioassay values for oxytocin and vasopressin.

It is quite evident that an almost total loss of all three materials can be obtained by severe dehydration procedures. It is also evident that about a 50 per cent reduction in the amount of all three materials can be obtained by alloxan treatment. A reduction in neurosecretion in the posterior lobe of alloxan diabetic rats has been reported previously (Kratzsch, 1951).

Earlier reports have indicated that there is a decrease in neurosecretion in the posterior lobe following adrenalectomy (Eichner, 1953; Melandra and Corbetta, 1953). Our data, to the contrary, indicate that some increase in both oxytocin and neurosecretion occurs at 7–10 days after adrenalectomy. In view of the errors in the assay methods these changes are probably not of much physiological significance. The maintenance of the animals on 1 per cent NaCl probably was important in preventing more dramatic changes.

It is seen in comparing the neurosecretion values for normal animals in figures I and II that a considerable difference exists. The only known difference in these two groups of animals was a slight difference in age and in body weight. They were killed at the same time of the year and the glands were processed and evaluated in the same manner. These values differ statistically and in recognition of this fact we must caution against basing major conclusions on differences of similar or smaller magnitude.

In connection with the changes accompanying lactation one has the problem of defining a suitable control. As control values for hormone levels we have used females which had borne 4 litters but which were not pregnant or lactating. In such animals the hormone levels are about double those of male rats of comparable size. For neurosecretion we have investigated other possible categories of control groups. It may be seen from the values given in figure III that either the non-lactating (N_1) or pregnant (N_2) group appears to constitute a suitable control for the lactating groups. A clear cut and progressive decline in the level of both oxytocin and vasopressin in the posterior lobe occurs during the course of lactation. We see no indication for a selective dis-

charge of oxytocin during lactation as claimed by Dicker and Tyler (1953). Taking either the non-lactating or pregnant female as the control a similar but quantitatively somewhat smaller decline in neurosecretion is seen during the course of lactation. This argues for a very close association of these three materials, at least insofar as their storage and release phases are concerned. In this connection, it should be pointed out that we have never observed neurosecretion within the vessels of the posterior lobe of the rat so that it seems doubtful if this material is actually secreted unmodified into the blood stream. What actually happens to this stainable material in the secretory process is a question that remains a fascinating enigma.

Summary

A correlative study of oxytocin, vasopressin, and Gomori-positive neurosecretion in male rats subjected to various treatments and in female rats during lactation has been made. The pituitary content of oxytocin and vasopressin was determined by bioassay while neurosecretion was evaluated in a semiquantitative manner on Zenker-formol fixed glands stained either with Gomori's chrome alum hematoxylin or aldehyde fuchsin method. The pituitary content of these materials was determined for groups of male rats subjected to the following: adrenalectomy, dehydration, alloxan, cortisone, and ACTH treatment. In general, it was found that there is a close correlation in the response of the hormones and neurosecretion to stimuli which alter the function of the posterior lobe. A maximal depletion of all three materials followed severe dehydration, whereas a decrease of about 50 per cent was seen in animals made diabetic by alloxan treatment. A progressive, marked decline in the pituitary content of oxytocin, vasopressin and neurosecretion was observed during the course of lactation.

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TUMORS INDUCED BY METHYLCHOLANTHRENE IN THE TURKEY[a]

R. H. RIGDON

Carcinogenic hydrocarbons have been used for the induction of tumors in the chicken (Knight and Taber, 1954, 1955; Oberling, *et al.*, 1936; Peacock and Peacock, 1949, 1954), in the pigeon and guinea fowl (Duran-Reynals and associates, 1945; Murphy and Sturm, 1946), in the dove (Lanza, 1941), in the duck (Duran-Reynals and associates, 1945; Rigdon, 1956a), and in the parakeet (Schlumberger, 1956). Attempts to produce tumors in the turkey have been unsuccessful (Duran-Reynals and associates, 1945; Lorenz, 1944).

Methods

Eighteen turkeys were used, 17 were broad-breasted bronzes crossed with Beltsville small whites, 5 weeks of age, and one was a Beltsville small white, 16 weeks old. A 0.25 per cent solution of methylcholanthrene in acetone was dropped onto the skin of the thorax beneath the right wing. Usually 1.0 or 2.0 ml. were applied daily except for the younger birds that received only 0.5 ml. The feathers were not plucked during the time of application of the carcinogen except in nine birds whose feathers were plucked only during the last two weeks of treatment. Nine of the turkeys were treated 32 times within a period of 100 days. Seven were treated 59 times within a period of 130 days. One bird was treated 82 times within a period of 250 days and one was treated 18 times within a period of 25 days. One bird was sacrificed on the 70th day after the first application of methylcholanthrene, one on the 194th day, five between the 215th and the 225th day, nine between the 300th and 350th day, and two between the 420th and 450th day. All the birds were autopsied immediately except for one (T 1130) that was dead for several hours before the autopsy. Five sections were removed from the treated and the untreated areas of skin. The sections were fixed in a 10 per cent solution of formalin. They were prepared in the routine manner for the paraffin technique and stained with hematoxylin and eosin.

Results

Varying-sized groups of lymphocytes were present in the dermis and subcutaneous tissues in the area of skin treated with the methylcholanthrene. Very few lymphocytes were observed in the normal skin (Fig. 1A). These groups of lymphocytes occurred

either in the wall or periphery of small blood vessels and capillaries (Fig. 1B). The number and size of the individual groups of lymphocytes in the skin of the 18 turkeys are given in table 1; 4+ indicates the maximum.

TABLE 1

Tumors in Skin of Turkeys Treated with Methylcholanthrene

Turkey No.	No. of Treatments	Days After Treatment		Lymphocytes in Dermis		
		First	Last	Treated	Control	Tumors
1125	59	194	45	+	0	No tumors
6507	18	70	56	+++	0	No tumors
6504	32	216	83	++++	0	No tumors
6501	32	219	86	++++	Very few	No tumors
6508	32	219	86	+++	Very few	No tumors
1124	59	330	86	++	0	Hemangioma
6622	32	221	88	++++	0	Lymphoma 1.0 cm.
6624	32	221	88	+++	0	Hemangioma
1127	59	310	171	Few	0	Papilloma
3181	82	421	172	+	0	No tumors
6630	32	305	172	++	Very few	No tumors
1130	59	316	177	Few	0	Papilloma
1129	59	325	185	++	0	Hemangioma
1126	59	327	187	++	0	Hemangioma
6513	32	334	206	+++	.	No tumors
6511	32	347	216	++	Very few	No tumors
6509	32	347	216	++	Very few	No tumors
1133	59	450	314	+	0	Hemangioma

The groups of lymphocytes sometimes coalesced. In turkey 6622 such a mass (Fig. 2) reached a diameter of 1.0 cms. and the overlying skin ulcerated. Usually these groups of lymphocytes were microscopic.

The largest number of lymphocytes was present in the skin of turkeys sacrificed from 50 to 90 days following the last application of methylcholanthrene (Table 1). The number of lymphocytes decreased with the increase of the interval between the last application of methylcholanthrene and death of the bird, as shown in table 1.

Hemangiomas were the most frequent tumor to occur in the methylcholanthrene-treated areas of skin. Five turkeys, as shown in table 1, developed one or more hemangiomas. The tumors usu-

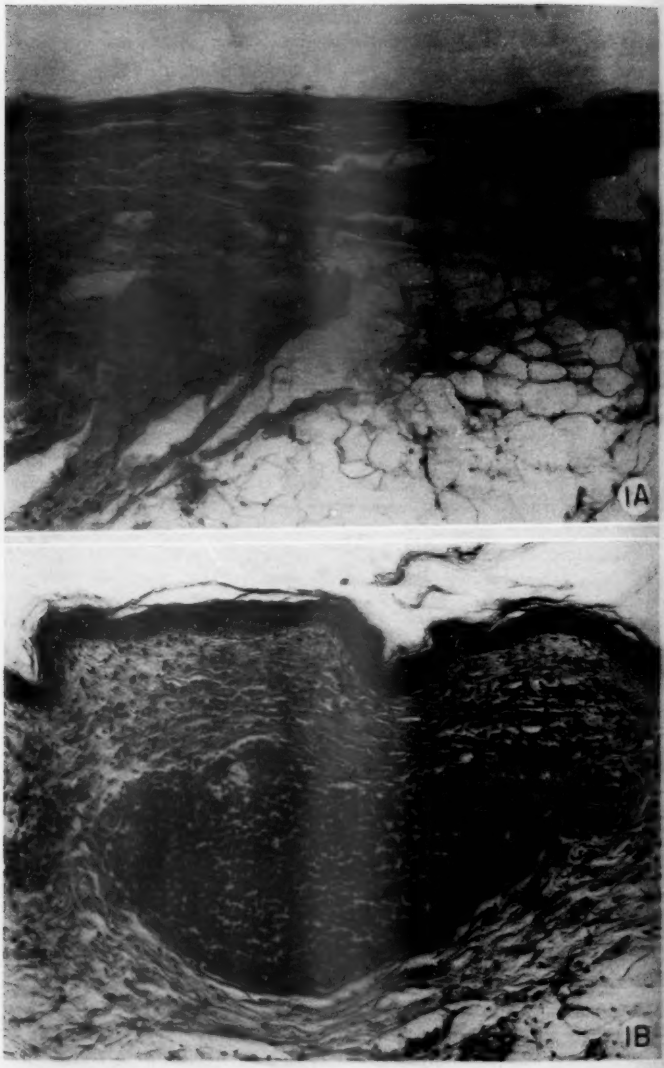

FIG. 1. T-6504: A. There are few if any lymphocytes in the skin of the turkey. B. In the area of skin treated with methylcholanthrene the cells in the epidermis have proliferated. Groups of lymphocytes occur in the dermis. These cells are present about the periphery of the capillaries and small blood vessels. H&E stain x 150.

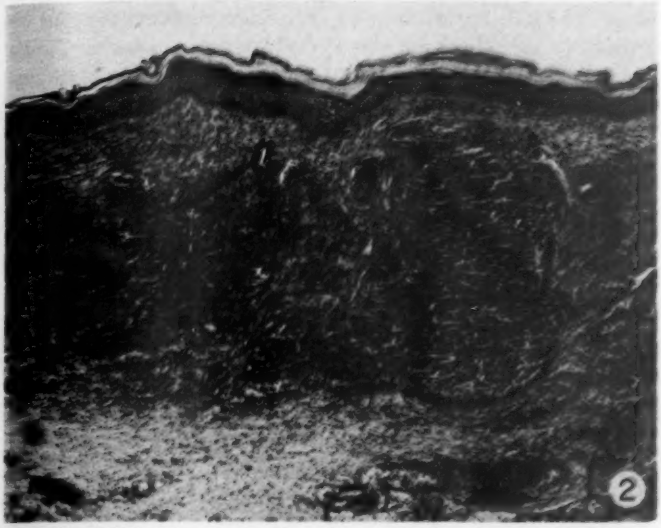

FIG. II. T-6622: The group of lymphocytes in the area of methylcholanthrene-treated skin coalesce to form a lymphoid tumor 1.0 cm. in diameter. H&E stain x 120.

FIG. III. T-1133: Hemangioma in the area of skin treated with methylcholanthrene.

ally were small, measuring 0.5 cm. or less in diameter (Fig. 3). Ordinarily they were not elevated above the skin surface. There was a marked variation in the size of the vascular channels in the different tumors (Fig. 4). These were lined by endothelial-like cells. Lymphocytes were conspicuous in and about many of the hemangiomas.

A papilloma developed in the methylcholanthrene-treated area of skin in only two turkeys, 1127 and 1130. These were typical papillomas.

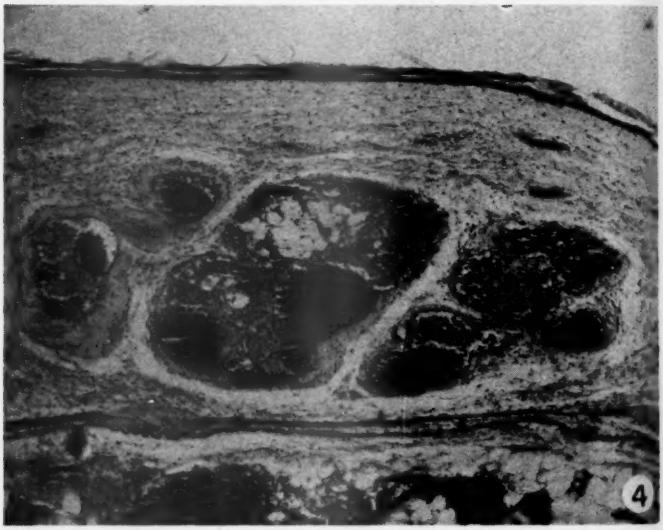

FIG. IV. T-1124: Hemangioma. Many of the large channels are filled with blood. H&E x 47.

Discussion

Few tumors develop in the skin of turkeys treated locally with multiple applications of a 0.25 per cent solution of methylcholanthrene in acetone. It is of interest, however, to find that papillomas and hemangiomas do occur following local applications of this carcinogen since tumors apparently have not been produced in the turkey (Duran-Reynals and associates, 1945; Lorenz, 1944). The papillomas and hemangiomas are typical and re-

semble the tumors occurring in the skin of the duck (Rigdon, 1956a). There is a marked increase in the number of hemangiomas and papillomas occurring in the skin of the duck as compared with the turkey when both are treated with the same carcinogen.

The local proliferation of lymphocytes in the skin of the turkey is similar to that previously described by Rigdon (1956b) in the skin of the chicken treated with this same carcinogen. Since numerous controls were used in the experimental study in the chicken, similar experiments were not repeated in the turkey. From our study in the chicken it was suggested that these groups of lymphocytes resulted from the stimulation by this carcinogen of a local cell, probably a histocyte, having the potentialities to form lymphocytes. Such a proliferation of lymphocytes apparently corresponds to the proliferation of either epithelial cells to form a papilloma or endothelial cells to form a hemangioma. The largest local group of lymphocytes observed in the skin of either the chicken, duck, or turkey occurred in the latter host.

The frequent association of lymphocytes and hemangiomas in the skin of the turkey, as well as in the duck and the chicken, treated with methylcholanthrene would support the opinion previously expressed (Rigdon *et al.*, 1956) that these lymphocytes and endothelial cells may originate from the same parent cell. A similar association between lymphocytes and hemangiomas has been observed by Burmester (1947) in lymphomatosis in the chick. He states, "There were only two cases (sixth passage) of angiomas, which did not show gross evidence of lymphoid involvement, and several in all passages which had lymphoid involvement without gross evidence of hemangiomatosis." Such lymphomatous lesions in fowls have been considered to be due to a virus; however, in this study in the turkey, it is of interest to find the groups of lymphocytes, the papillomas and the hemangiomas occurring only in the methylcholanthrene-treated areas of skin. Since numerous experimental observations have indicated the role of chemical carcinogens in the etiology of skin tumors, it would seem preferable at this time to consider these tumors in the turkey to be directly related to methylcholanthrene rather than produced by an unknown virus as some virologists no doubt will postulate.

Summary

Hemangiomas and papillomas have occurred in the skin of turkeys following local applications of a 0.25 per cent solution of methylcholanthrene in acetone.

Local proliferations of lymphocytes have occurred in the methylcholanthrene-treated areas of turkey skin. This lesion is identical with that previously described in the skin of chickens treated with the same carcinogen.

The tumorigenic response of the turkey skin to methylcholanthrene is much less than that observed in the duck and the chicken.

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STUDIES ON ASCORBIC ACID IN BLOOD. II. STUDIES ON ASCORBIC ACID IN ERYTHROCYTES AND LEUCOCYTES NOTES ON HEPATIC ASCORBIC ACID

LOTHAR L. SALOMON AND JOSEPH E. JOHNSON[a]

During a continuing program aimed at investigating aspects of the metabolism of ascorbic acid, it became probable that erythrocytes of rats contain two forms of reduced ascorbic acid (4). This deduction was based on measurements of the rate of exchange of ascorbic acid between erythrocytes and plasma, employing the C^{14}-labeled vitamin. Rapid exchange occurred, and the specific activity of cellular ascorbic acid passed through a maximum, but never equaled that of ascorbic acid in plasma during the 50 minutes following injection of labeled ascorbic acid. It was concluded that exchange must be incomplete, one fraction of cellular ascorbic acid exchanging more slowly and, therefore, accumulating radioactivity more slowly, than another smaller fraction. Behavior of this nature required the existence of a bimodal specific activity-time curve (4). By proper choice of experimental conditions, it has now been possible to locate the second maximum, thus confirming previous interpretations.

Rats are known to be independent of exogenous sources of vitamin C. Nevertheless, there is reason to believe that not all tissues of the rat may be able to synthesize the vitamin (5). However, the present data indicate that neogenesis of ascorbic acid may occur in erythrocytes of rats.

Concurrently, ascorbic acid in liver and leucocytes of rats was also investigated. Ascorbic acid in the liver exchanges rapidly with extra-cellular ascorbic acid, while the exchange is somewhat slower in leucocytes. The data strongly suggest the occurrence of ascorbic acid neogenesis in leucocytes. Liver of rats is known to produce ascorbic acid (2), but the present results do not permit evaluation of the rate of such synthesis except as a rough approximation, since exchange of intracellular with extracellular vitamin is quite rapid. In connection with these studies, a method for the evaluation of steady-state flow and neogenesis of ascorbic acid is presented.

Methods and Materials

Animals, methods and materials used, and the determination of specific activity of ascorbic acid in plasma and erythrocytes were previously described (4). Substantially the same procedure as for plasma and erythrocytes was used in the case of leucocytes and livers. For the determination of radioactivity and concentration of ascorbic acid in the latter materials, homogenates of pooled samples were usually employed. While a certain loss of information results from using pooled samples, the exigencies of the situation were such as to make this procedure unavoidable.

Previously, the specific activities of ascorbic acid in erythrocytes were corrected for the presence of intercellular plasma (4). For the present data, this was not done. Correction would not have resulted in significant change in values, since the specific activities of ascorbic acid in plasma and cells were similar.

Leucocyte preparations were visibly contaminated with erythrocytes. Because of the relatively high concentration of ascorbic acid, approximately 30 to 40 times that in erythrocytes, it is improbable that the error in specific activities for ascorbic acid in leucocytes is appreciable even when contamination is large.[b] The fact that maximal specific activity for ascorbic acid in the two types of cells was achieved at widely different times, supports this belief. However, additional experiments to study liver and leucocytes with better specimens are planned, and data presented for these tissues are to be considered as preliminary information.

Results and Discussion[c]

If it is assumed that a steady state with respect to all reactions involving cellular ascorbic acid exists, the net rate of change in concentration of cellular ascorbic acid may be expressed by

$$(1)\quad \frac{dA_c}{dt} = a + b - (a + b) = 0$$

since the rate of inflow, b, plus the rate of synthesis *in situ*, a, must be counterbalanced by a sum of rates, $a + b$, which are a measure of the total loss of cellular ascorbic acid, whatever the routes. If inflowing radioactive ascorbic acid, and newly-synthesized nonradioactive ascorbic acid, both mix uniformly with

the exchanging fraction of cellular ascorbic acid, then the net rate of change in radioactivity in the tissues can be expressed by

$$(2)\quad \frac{dR_c}{dt}=br_p-(a+b)r_c=A_c\frac{dr_c}{dt}.$$

When r_c reaches a maximum, $dr_c/dt=0$, and

$$(3)\quad a=\frac{b\ (r_{pm}-r_{cm})}{r_{cm}}$$

so that the rate of synthesis may be evaluated under favorable circumstances if b becomes known. It may be noted that, if r_{pm} should equal r_{cm}, a is zero. However, experimental conditions may not permit such a definitive statement. If b is large relative to a, it may appear as though r_{pm} and r_{cm} were equal. This fact must be borne in mind.

By substitution of equation (3) into equation (2), one obtains

$$(4)\quad A_c\frac{dr_c}{dt}=br_p-br_c\left(\frac{r_{pm}-r_{cm}}{r_{cm}}\right)-br_c$$

and, after rearranging,

$$(5)\quad b=\frac{A_c\int dr_c}{\int r_p dt-\frac{r_{pm}}{r_{cm}}\int r_c dt}$$

Graphical evaluation of the integrals, which can be achieved by using the curves of figure I, permits calculation of the rate b; substitution of b, and the values for r_{pm} and r_{cm} from figure I, into equation (3), permits estimation of a.

Should r_{pm} and r_{cm} be equal, equation (5) simplifies to equation (4) of another paper (4), where certain limiting conditions which must apply in the use of these equations have been discussed. Further simplification is possible when r_p is deliberately held constant throughout the experimental period. This will be of further consequence in data to be reported in the future. It is apparent, of course, that these equations are not specifically restricted in use to the current data.

Curve B, figure I, and table 1, which describes the changing specific activity of hepatic ascorbic acid in rats following administration of 1.0 mg. of ascorbic-1-C^{14} acid, represents a case where

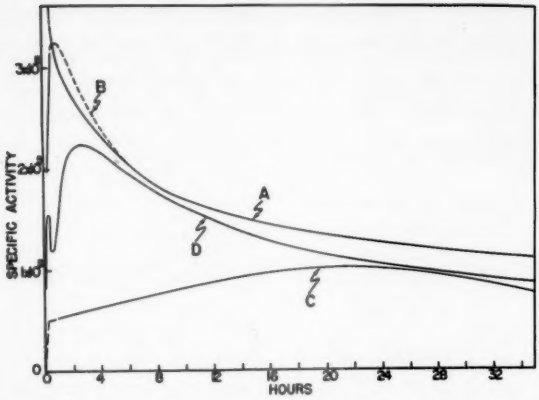

FIG. I. Specific activity of ascorbic acid in plasma (A), liver (B), leucocytes (C), and erythrocytes (D). These rats received 1.0 mg. of ascorbic-1-C^{14} acid (3.4 μc) by intraperitoneal injection. Time of injection is "zero time." Smooth curves constructed from data of Table 1.

rate of flow is so rapid relative to rate of synthesis, that the latter cannot be measured. It is known, however, that synthesis does take place in this tissue (2). Moreover, there is no clearcut evidence for the existence of separate forms of ascorbic acid, rapidly- and slowly-exchanging with extracellular ascorbic acid, as there is for adrenal tissue (6), erythrocytes (4) (see also below), and probably also leucocytes (see below). This does not preclude the existence of two forms of ascorbic acid in hepatic tissue, but can be safely interpreted only in that both forms may exchange at rapid rates. An approximation of the rate of flow can be made, by neglecting the rate of synthesis, and using equation (5), or equation (4) (4). The rate of flow of ascorbic acid into liver tissue is found to be 9×10^{-1} mg. per min. per 100 gm. of tissue. Since neogenesis proceeding at about one-fifth the rate of inflow is still discernible (see data for erythrocytes below), it appears that the rate of neogenesis is not more than roughly 1×10^{-1} mg. per min. per 100 gm. of tissue. The tentativeness of these calculations need not be further emphasized.

Curve C, figure I, and table 1, describe data for leucocytes. The rapid initial increase, followed by a slow subsequent rise in specific activity, indicates that ascorbic acid in such cells also occurs in two forms. In this case, however, even the second maximum

Specific Activity of Ascorbic Acid in Plasma, Erythrocytes, Leucocytes, and Liver of Rats After Injection of 1.00 mg. of Ascorbic-1-C¹⁴ Acid

Time	15 min.	35 min.	50 min.	2.5 hrs.	3.5 hrs.	4.5 hrs.	8.5 hrs.	16 hrs.	21 hrs.	34 hrs.
	...	...	...	301	231	289	144	117	149	113
	...	...	...	189	241	279	154	124	132	94
	...	...	...	226	236	208	228	128	115	102
Plasma	...	...	...	260	237	257	200	175	126	136
	...	...	...	253	211	271	163	108	125	95
	...	...	...	...	...	...	199	138	172	133
	...	...	...	...	...	...	142	138	161	112
	...	...	...	...	...	...	...	154	139	...
	...	...	...	...	...	...	...	182	133	...
Average	630	371	312	245	231	231	176	141	139	112
	...	...	...	282	...	175	218	126	81	63
	...	...	...	187	...	191	156	96	143	58
	...	...	...	198	...	217	208	83	126	78
Erythrocytes	...	...	...	243	...	203	115	154	137	103
	...	...	...	210	...	259	...	95	109	69
	...	...	...	...	...	...	...	147	...	99
	...	...	...	...	...	...	...	118	...	88
	...	...	...	...	...	...	...	147	...	...
Average	142	126	137	224	...	2 0	174	121	119	80
	56(3)	50(3)	54(2)	67(4)	53(5)	48(2)	82(4)	69(3)	92	74(3)
Leucocytes	...	39(3)	49(2)	...	...	74(3)	...	88(2)	104(4)	82(4)
	...	...	...	...	...	...	...	114(2)	...	...
Average	56	45	52	67	53	64	82	87	102	79
	230(3)	313(3)	...	263(4)	198(5)	252(3)	165(4)	147(2)	155(4)	104(4)
	...	...	...	...	...	295(2)	...	186(2)	126	82(3)
Liver	...	...	...	...	...	...	...	127(3)	152	86
	...	...	...	...	...	...	...	154	...	...
Average	230	313	...	263	198	269	165	150	150	94

Specific activities expressed as counts per min. per mg. of ascorbic acid $\times 10^{-3}$. Corrected for background and self-absorption, but not absolute. Averages weighted where applicable. Data covering the 15 minute, 35 minute and 50 minute periods for plasma and erythrocytes have been previously shown (4). Numbers in parentheses following specific activity values indicate pooled sample composed of number of specimens shown.

lies well below the corresponding specific activity of ascorbic acid in plasma, and curves A and C do not intersect. The interpretation we place on this, as well as the analogous situation existing for erythrocytes (curve D), is that neogenesis of ascorbic acid takes place in leucocytes and erythrocytes. Constant addition of nonradioactive, newly-formed ascorbic acid at a rate significant in comparison with the rate of inflow, may reasonably be assumed to maintain the specific activity of cellular ascorbic acid at levels below the corresponding specific activity of extracellular ascorbic acid at all times.

Estimation of the rate of inflow and the rate of synthesis may now be undertaken. It is to be noted that it would be difficult indeed to determine the net rate of synthesis by means other than such as are here described. Although the slowly-exchanging fraction may derive its radioactivity either from the rapidly-exchanging fraction, or directly from extracellular ascorbic acid, this does not matter for the present purposes (6). The specific activity of inflowing ascorbic acid must either be r_p, or else be very similar to r_p. We hope to clarify this in the future. Using equations (3) and (5) above, the rate of flow of ascorbic acid into leucocytes is found to be approximately 3×10^{-2} mg. per min. per 100 gm., the rate of synthesis about 9×10^{-3} mg. per min. per 100 gm. of cells and, consequently, the rate of loss or outflow must be the sum of these two values. In the scurvy-liable species, leucocytes maintain appreciable levels of vitamin C during deprivation for longer periods than other tissues which have been investigated (1), even when the concentration in plasma has fallen to values difficult to measure. It might be speculated that leucocytes of such species retain a degree of ability to synthesize ascorbic acid, or that they must differ from leucocytes of rats in some other property. On the other hand, perhaps all that is involved is a pronounced ability to concentrate ascorbic acid so long as traces remain in plasma. The rate of increase in specific activity of ascorbic acid in these cells is slow, even when neogenesis is taken into account. This is chiefly the result of the relatively high concentration of cellular ascorbic acid, rather than failure of radioactivity to move across the cellular membrane, and thus does not indicate any special tenacity with which these cells retain the vitamin.

The rapidly-exchanging fraction of ascorbic acid in erythrocytes quickly attains maximal specific activity after injection of

labeled ascorbic acid (4). The slowly-exchanging fraction has been found to come to a maximal value approximately 2.5 hours after injection, as shown by curve D, figure I. It is exceeded in that respect only by ascorbic acid in liver, among the tissues studied. By the methods described above, the rate of inflow of ascorbic acid has been determined to be 3×10^{-3} mg. per min. per 100 gm., and the rate of synthesis 6×10^{-4} mg. per min. per 100 gm. of cells. The rate of synthesis is appreciably lower than the rate of inflow, but still produces a discernible effect on curve D. It may therefore be estimated that the ratio of rate of neogenesis to rate of inflow must be smaller in hepatic tissue than in erythrocytes.

Summary

Confirming evidence for the existence of two kinds of ascorbic acid in erythrocytes has been obtained. The evidence also indicates that erythrocytes of rats may be able to synthesize ascorbic acid. The rate at which ascorbic acid flows into the slowly-exchanging fraction, and the apparent rate of synthesis, have been estimated.

Leucocytes of rats also seem to contain rapidly- and slowly-exchanging ascorbic acid, and are probably capable of synthesizing the vitamin at an appreciable rate.

Hepatic ascorbic acid comes to maximal specific activity so rapidly that measurement of the rates of flow and synthesis could only be made as an approximation.

These experiments depended upon analysis of rate data obtained *in vivo* with ascorbic-1-C^{14} acid.

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[a] From the Department of Biochemistry and Nutrition, The University of Texas Medical Branch, Galveston, Texas. This research was supported in part by generous grants from the United States Public Health Service, and the National Science Foundation. Aid by granting a McLaughlin Medical Student Fellowship to one of us (J. E. J.) is gratefully acknowledged.

[b] Ludány and Megay (3) report the concentration of ascorbic acid in leucocytes of rats to be 20 to 25 mg. per cent. In our preparations, variable lower values, indicating as much as 50 per cent admixture of erythrocytes and plasma, were observed. For purposes of calculating rates, the concentration of ascorbic acid in leucocytes will be assumed to be 25 mg. per 100 gm. of cells. Appreciable error in specific activity is unlikely, except possibly during the very early periods after injection, and will be disregarded.

[c] The following symbols will be used:

A_c = concentration of cellular ascorbic acid. It is 0.45 mg. per 100 gm. of erythrocytes (slowly-exchanging) (*4*), 25.8 mg. per 100 gm. of liver (53 specimens, some pooled, range 20.0 to 28.8 mg., both extremes found in single specimens).

For leucocytes, see note b.

t = time

a = rate of synthesis of ascorbic acid in steady state.

b = rate of inflow of ascorbic acid in steady state.

R_c = cellular radioactivity, c. p. m. per unit weight of tissue.

r_c and r_p = specific activity of ascorbic acid in cells and plasma, respectively, in c. p. m. per mg. of ascorbic acid.

r_{cm} and r_{pm} = maximal specific activity of cellular ascorbic acid, and specific activity of ascorbic acid in plasma measured at the time of that event, respectively.

[d] Limits for graphical integration: Liver, peak to 7 hours; leucocytes, 4 to 34 hours; erythrocytes, 2.5 to 34 hours. Periods were so chosen to avoid regions of uncertainty in curves, and to avoid segments where rapidly-exchanging ascorbic acid constitutes too large a proportion of the average specific activity.

STUDIES ON ADRENAL ASCORBIC ACID. IV. SLOW PHASE OF EXCHANGE WITH EXTRACELLULAR ASCORBIC ACID

LOTHAR L. SALOMON[a]

In preceding papers, it was demonstrated that radioactivity accumulated rapidly in adrenal glands of rats following administration of labeled ascorbic acid (3), and that this radioactivity could be identified as unchanged ascorbic acid (2). Furthermore, synthesis of ascorbic acid in these glands proceeded either at a very slow rate, or not at all (2). In measuring the changing specific activity of adrenal ascorbic acid at intervals during the 50 minutes following injection of labeled ascorbic acid, it was discovered that exchange with extracellular ascorbic acid was apparently incomplete (3). The existence of two "kinds" of adrenal ascorbic acid was postulated, which were suggested to differ in the rate of exchange with ascorbic acid in plasma, as well as in the effect which ACTH had upon them.

By extension of this reasoning, it was concluded that a second maximum in the specific activity-time curve for adrenal ascorbic acid should occur later in time. Consequently, the first maximum, representing attainment of equality between specific activities of adrenal rapidly-exchanging and plasma ascorbic acid, should be followed by a second maximum representing similar equality between adrenal slowly-exchanging and rapidly-exchanging (or plasma) ascorbic acid. Data presented below are in support of this, and lend further weight to the original conclusions.

Methods and Materials

All animals were male, hypophysectomized rats of the Sprague-Dawley strain. None received ACTH unless otherwise indicated below. In all respects, including dosage of ascorbic-1-C^{14} acid and ACTH, method of administration of these substances, and treatment of animals and samples, procedures remained unchanged (3), except as noted.

Results and Discussion

During the 50 minutes following injection of labeled ascorbic acid, the specific activity of adrenal ascorbic acid of hypophysectomized rats was found to increase rapidly, passing through a maximal value (3). However, even at that point, the observed specific activity never equaled that of ascorbic acid in plasma. This was attributed to incomplete exchange, *i.e.*, a part of adrenal ascorbic acid did not measurably participate in exchange with radioactive extracellular ascorbic acid under the experimental conditions. Provided that this latter fraction, which was presumed responsible for the depression of the observed maximal value of specific activity, accumulates appreciable amounts of radioactivity under less acute experimental conditions, it may be anticipated that a second maximum in the specific activity-time curve for adrenal ascorbic acid should be detectable. Figure I, curve B, which is a smooth curve constructed from the data of table 1, supports this idea. It demonstrates the existence of such a maximum, about 8.5 hours after injection of labeled compound, the first maximum having occurred in about 20 minutes. It is believed that this constitutes reasonably conclusive evidence for

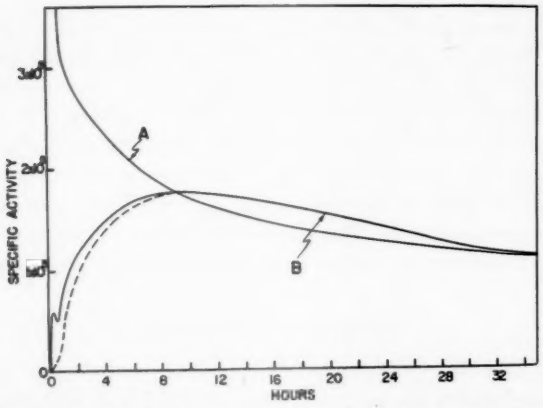

FIG. I. Specific activity of ascorbic acid in plasma (A) and adrenal glands (B). Rats received 1.00 mg. of ascorbic-1-C^{14} acid (3.4 μc) by intraperitoneal injection. Time of injection is "zero time." Smooth curve for adrenal ascorbic acid constructed from data of table 1; plasma data shown elsewhere (5). Segment of curve B shown dashed represents specific activity of slowly-exchanging ascorbic acid (see note c).

TABLE 1

Specific Activity of Ascorbic Acid in Adrenal Glands of Rats after Injection of Labeled Ascorbic Acid

	Time						
	50 min.	2.5 hrs.	4.5 hrs.	8.5 hrs.	16 hrs.	21 hrs.	34 hrs.
	79	137(4)	175(3)	159	145	166	102
	85		112	155	149	170(4)	87
	69		125	206	156	134	106
	80			176(4)	174(2)	169	99
	100				176(2)	169	105
	77				151	154	
					131		
					124		
					183		
Average	82	137	152	175	158	164	108

Corresponding specific activities for ascorbic acid in plasma are shown elsewhere (5). Specific activities in counts per min. per mg. of ascorbic acid $\times 10^{-3}$, corrected for background and self-absorption, but not absolute. Numbers in parentheses indicate pooled sample composed of number of specimens shown. Averages weighted where applicable.

the existence of two distinct kinds of adrenal ascorbic acid, which differ not only in respect to the rates at which they accumulate radioactivity, but more importantly, in respect to their response to ACTH. Aspects of the latter point have been discussed (3).

In an earlier series of experiments, it was shown that ACTH decreased the amount of ascorbic acid per unit weight of adrenal tissue, but not radioactivity per unit weight, thus effecting an increase in specific activity (3). Presumably, this was the result of the hormone's action being directed solely at the substantially untagged, slowly-exchanging form of adrenal ascorbic acid. It is then to be expected that, when the slowly-exchanging form attains a level of specific activity which does not differ a great deal from that of rapidly-exchanging ascorbic acid, the hormone should not produce a significant change in observed (average) specific activity, in spite of its effect on concentration of adrenal ascorbic acid. This expectation was also confirmed. Twenty-one hours after administration of ascorbic-1-C^{14} acid, the ratio of the specific activities of ascorbic acid in adrenal glands to that in plasma averaged 1.17; for two rats which received 3 I. U. of ACTH intraperitoneally, the ratios were 1.27 and 1.45, respectively, accompanied by a 43.7 per cent decrease in concentration of ascorbic acid. In 5 intact rats, the ratio was 1.33 on the average, 8.5 hours after introduction of labeled compound, and 1.35 in 4

intact rats which had received ACTH, depressing the concentration of ascorbic acid by 26.5 per cent. This bears out the contention discussed above. However, it raises another point which must be further investigated, namely the reason why that ratio is nearly 1.0 at the 8.5 hour period for hypophysectomized rats, but much higher for intact rats.[d] It may be that the rate of exchange of the slowly-exchanging fraction is increased by hypophysectomy. It may also be that the stress incidental to handling and injection produced a small decrease in slowly-exchanging ascorbic acid in intact rats. Reconstitution of this ascorbic acid would have to occur from then highly radioactive extracellular ascorbic acid, so that even a small excess deposition of extracellular ascorbic acid would result in a marked increase in specific activity of intracellular ascorbic acid.

For purposes of calculating the steady-state rate of exchange of adrenal slowly-exchanging ascorbic acid, it will not be necessary to make a decision whether exchange involves the reaction: ascorbic acid (adrenal, rapidly-exchanging) $\rightleftarrows$ ascorbic acid (adrenal, slowly-exchanging), or the reaction: ascorbic acid (extracellular) $\rightleftarrows$ ascorbic acid (slowly-exchanging). It may be assumed that rapidly-exchanging ascorbic acid has a specific activity essentially the same as that of extracellular ascorbic acid during the periods here considered, because of the rapid rate of exchange between them. Aspects of this assumption, which would be patently erroneous only shortly after administration of labeled ascorbic acid, have been discussed (3).

It should be emphasized that no inferences are being made regarding the mechanism of the reactions involved (nor regarding the meaning of the terms "free" and "bound ascorbic acid" except as it involves the rate of exchange with extracellular ascorbic acid) here, or in other papers (3, 4, 5). These points will be discussed in the future in the light of other evidence. For the present, it may be noted that "rate of exchange" refers to the steady state. When a steady state exists, as it does here (2), then the rate at which ascorbic acid is incorporated into the slowly-exchanging fraction must be equal to the loss from that fraction. It is inconsequential for the moment whether loss involves conversion to rapidly-exchanging or plasma ascorbic acid, or involves more radical transformations, as oxidation. Hence,[b]

$$(1)\quad \frac{dA_c}{dt}=K_{gain}-K_{loss}=0$$

and $$(2)\quad K_2=K_{gain}=K_{loss}.$$

Since the rate of gain of radioactivity by slowly-exchanging ascorbic acid can then be represented by K_2r_p, and the rate of loss by K_2r_c, the net rate of change in radioactivity becomes

$$(3)\quad \frac{dR_c}{dt}=K_2\,(r_p-r_c)=A_c\frac{dr_c}{dt}.$$

This is the equation as used before (3, 4), and the same restrictions in its use apply here. K_2 can be evaluated by graphical integration of this equation (4). The average concentration of adrenal slowly-exchanging ascorbic acid being 2.84 mg. per gm. of tissue (3), one finds by integrating that K_2 is 8.7×10^{-3} mg. of ascorbic acid per min. per gm. of adrenal tissue. This is about one-third the rate at which the rapidly-exchanging form gains and loses ascorbic acid. The delay in attainment of the second maximum is thus attributable to slow exchange only in part. A major contributory factor is the relatively high concentration of slowly-exchanging ascorbic acid in adrenal glands.

Summary

Confirmation of the previously proposed existence of two kinds of adrenal ascorbic acid has been adduced. Exchange of adrenal with radioactive plasma ascorbic acid proceeds in such a way that two distinct maxima in specific activity of adrenal ascorbic acid occur. One of the maxima occurs about 20 minutes after injection of labeled ascorbic acid, the other 8.5 hours after injection.

It has also been shown that the effects of ACTH on the adrenal gland, which includes an action specifically directed at the slowly-exchanging fraction, do not alter the specific activity when slowly- and rapidly-exchanging fractions have become labeled to about equal extents. This is in contrast to the hormonal effect during the early periods after administration of ascorbic-1-C^{14} acid, when the specific activity was found to be markedly altered.

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[b] The symbols used are essentially those used previously (4).

[c] The dashed curve in figure I represents the specific activities of slowly-exchanging ascorbic acid, rather than an average for adrenal ascorbic acid. This was arrived at by correcting for the contribution of rapidly-exchanging ascorbic acid to the average values: $(3.20 \times r_{c,\,av} - 0.36 \times r_p)/2.84 = r_{c,\,corr.}$ where 3.20 is the concentration of adrenal ascorbic acid, and 0.36 and 2.84 the concentrations of slowly- and rapidly-exchanging ascorbic acid, respectively, in mg. per gm. of tissue. Beyond the 8.5 hour period, the correction is small, and has been disregarded. Substantially the same value for K_2 is obtained by integrating between 8.5 and 34 hours, as between 1 and 34 hours, provided the corrected curve is used.

[d] Preliminary experiments have subsequently shown that these ratios may be too high, and that differences between intact and hypophysectomized rats may be small or non-existant in this respect. However, the conclusion regarding the effect of the hormone on specific activities remains unchanged.

A DEVELOPMENTAL STUDY OF THE FOURTH CRANIAL NERVE

JOHN G. SINCLAIR[a]

The fourth cranial nerve is unique among motor nerves of vertebrates in its dorsal course and complete decussation. This study attempts to follow the nerve from its nuclear origins and discusses some of the problems raised by its course, composition and function. New observations are interspersed in the running account and illustrated to a limited extent.

Materials and Methods

Embryo series of rat, mouse, and pig at several stages were available. Porpoise embryos (Prodelphinius coerulealbus) of 25, 35, 55, and 76 mm. length were obtained through the kindness of Professor Ogawa of Tokyo and series were prepared with iron hematoxylin staining. Human embryos of 4.5, 6, 13, 20, 35, 45 and 65 mm. were stained with hematoxylin and eosin. One 20 mm. series was prepared with the Hortega siver technique. Mouse embryos with open brains were produced by transplacental urethane injections (44). Several stages from 11 to 19 days gestation were sectioned. Adult human brains furnished blocks for binocular study of nerves and for transverse and sagittal series of the isthmus region using Castro's silver and Kluver's stain for both cells and fibers. The fourth nerve was sectioned near the brain and at the orbital entrance and osmicated.

Closure of the Neural Tube. Neural Crest of the Midbrain

Closure of the medullary plate in mammals begins in the midbrain region and at the time of closure there is little sign of neural crest here although it appears both cranial and caudal to this level. In the 11 somite chick a sheet of basophil cells from the forebrain descends immediately behind the optic vesicles to meet the prechordal mesenchyme arising from the preoral gut. The two then become inseparable. Both mandibular arch and ocular muscles are derived from this mesenchyme (2, 4, 5, 21, 22, 49). From the midbrain back to the otocyst the crest is abundant and is mostly taken into the trigeminal complex. Its history in the region of the midbrain is in dispute. The neural folds in closing here form a broad union with complete cell continuity (6). In

the adjacent forebrain and hindbrain only an ependymal roof-plate remains. Neural crest cells have been found bridging the midline in a 10 somite human embryo (9). In Squallus the crest forms a continuous fold from the lateral margin of the closing plate (15). The question arises concerning possible permanent inclusion of neural crest components in the midbrain region. Piatt 1909 found that removal of all obvious neural crest did not prevent the mesencephalic fifth nucleus from forming, but did reduce it considerably in amphibia. In recent studies of the origin of pigment cells in amphibia (32) it has been found that removal of the neural crest in most regions removes all potential melanoblasts, xanthoblasts, and guanoblasts except in the midbrain region. Here it was necessary to remove the medullary plate to some depth. The same was true for the medullary plate of the post coelomic region of the embryo. Neural crest in successive waves produces the several types of pigment cells and the sheath cells for peripheral nerves and ganglia. It seems entirely possible that such included cells could be responsible for the cells of the locus coeruleus, the mesencephalic fifth nucleus and other cells that follow the border of the brachium conjunctivum ventrally to the red nucleus and substantia nigra. It is also possible that small numbers of neural crest cells may remain outside and migrate along the course of peripheral nerves, forming at least the sheath cells of these nerves. Several authors have agreed that neural crest cells form no part of cranial nerve ganglia (8). In the spinal cord no epidermal placodes form and neural crest seems to form both ganglion cells and sheath cells.

Development of the basal lamina

Complete uniformity of cell columns in the lateral walls is the primary condition of the neural tube. Differentiation of motor cells begins with the extrusion of neuroblasts at certain points into the mantle layer. Extensive movements of neuroblasts follow and alter the relative positions of these motor masses. In the hindbrain these movements are largely lateral displacements of the branchial components. In the midbrain and isthmus there is some lateral shifting especially in the caudal part of the third and much of the fourth nucleus. This shift is not marked by the course of

the nerve as in the seventh, ninth and tenth nerves, but is more like the fifth nucleus which is displaced by the expansion of medial cell masses. Supratrochlear nuclei and expansion of the raphe operate at the level of the fourth nuclei.

The formation of the isthmus involves considerable elongation between dorsad migration of cerebellar vermis and the extension of crossed fibers in the smooth mesocoel roof during formation of the inferior colliculi. This then stretches out the fourth nucleus moving the superior portion rostrad to contact the third nucleus and breaking the remainder into one or two pairs between the superior pair and the level of the isthmus. The presence of accessory nuclei varies widely among mammals (28). The separation is already evident in the 13 mm. pig and human embryos. Expansion of the pons separates them from the fifth nucleus.

As soon as it is possible to distinguish the fourth nuclei it is possible to follow a clear path dorsally toward the anterior medullary velum, which in the embryo is characteristically thick. Windle (48) has seen dorsally directed fibers as early as the 6 mm. human embryo and a decussation by the 10 mm. stage (12). The question arises whether the fibers from the fourth nucleus are following a preformed path to the decussation or originate that path in their extension. Barron (7) shows that cells of the accessory nucleus start as the most ventral group and their axons follow the border of the marginal zone. Larsell (29) pointed out that in Lamprey larvae neuroblasts are distinguishable when the neucleus lies at the margin of the medullary velum and moves ventrally from this position. He also shows a stage in which the fibers are found on the same side as the nucleus and exiting without decussation. Martin (31) thought that neuroblasts in cat embryos had migrated across the midline to form a decussation. Before the isthmus lengthens the mesocoel roof overhangs the cerebellar primordium. The fourth nucleus at this time could be said to be in the cerebellum (2). In the formation of the vermis cerebelli there are massive movements across the midline and such migrations are similar to Martin's postulated shift of the fourth nucleus. These observations are neither supported nor denied by others. No reason, teleological or mechanical, has been advanced to explain this decussation.

Interpretation of the neuromeres

Von Baer in 1828 first described the scalloped depressions in the floor of the hindbrain of the embryo. In 1887 Orr named them neuromeres. McClure (30) enumerated ten of them including four in the midbrain. Hill extended the concept to ripples pictured on the medullary plate of the chick throughout its length. Arthur Keith in 1913, using a figure by Low, pictured the external ridges on a human embryo including the thalamus. Streeter in the Keibel and Mall Human Embryology limited the neuromeres to six in the hindbrain and his description has been generally followed.

The concept has been confused with the problem of the segmental origin of the head of which the neuromeres were supposed to be relicts. But neuromeres are always secondary to myotomes and other masses. Streeter related them to branchial nerves but admitted that the sixth nerve and its neuromere constituted an anomaly (Fig. 1). It has been a source of endless confusion to

FIG. 1. Neuromeres from the brain of a 6 mm. human embryo. Motor nuclei originating in each neuromere are numbered.

medical students that the branchial series has been labelled visceral. They cannot see that the twelfth is any less visceral than the fifth. The term visceral has been linked to the two neuron autonomic chain so thoroughly that it should not be applied to the branchial nerves. If we regard the motor series to striated muscle as one series the difficulties disappear (Figs. 1, 2). There is a neuromere for each one except the twelfth which is elongated over several segments like the motor nuclei of spinal nerves.

In the light of Sauer's analysis (41, 42) of the germinal zone of the neural tube the neuromeres gain significance. During the period of proliferation, in which the basal plate precedes the alar plate, all cells capable of mitosis remain tall columnar in form with tapering ends, continuously attached to the limiting membrane of the lumen (19). The resting and prophase nuclei occupy various depths of the germinal layer but only the metaphase and anaphase are seen in the clear inner zone commonly misnamed the ependymal zone. During this period the tapered form of the cells produces a depression in the surface opposite the most rapid mitosis, and a ridged margin where the production of cells is zero (Fig. 1). Once the cells are detached they move into the mantle layer. Here they grow in size, develop dendrites, become separated by interstitial glial material, and thereby press outward on surrounding structures. This erases and everts the neuromeres and constricts the central canal. Bulges also appear externally. By this time mitosis has ceased in the basal plate and is transferred to the alar plate where the process is repeated but not on a small segmental plan. There are expansions for the first, second, and combined fifth and eighth cranial nerves. The general expansion of the alar plate leaves the sulcus limitans as a nonproductive line of division. By this stage, however, it is a depression. In the isthmus the sulcus limitans rises to the margin of the medullary velum.

The fact that the fourth nerve produces a neuromere is indicated in figures 1, 2. The cephalic flexure, together with the growth in length of the whole isthmus and midbrain soon erases the margins. The motor nuclei are displaced from a position lateral to the canal to a ventrolateral position between 10 and 20 mm. stages. Mitosis in the fourth neuromere seen in a 13 mm. stage is dorsal to the differentiating nuclei and is probably producing cells destined for the supratrochlear nucleus. The third neuromere can be seen only in frontal sections of earlier embryos. It is transient and shallow.

The fourth nerve nuclei as Olszewsky (33) pictures them, consist of three pairs of cell masses. The principal nucleus is just caudal to the third nucleus and, except for the strikingly different path taken by the fibers, could not be separated from it. The remaining pairs are small and are not easily recognizable. Some of the cells are embedded in the surface of the medial longitudinal

fasiculus at some distance from the central canal and others are spread laterally. They are not united across the raphe by dendrites as are the cells of the third nucleus, but send their dendrites directly ventrad into the deeper parts of the fasiculus and more tortuously dorsad into the supratrochlear nucleus. This latter nucleus is composed of numerous large and small cells aggregated above each motor nucleus and oriented mostly in a longitudinal direction. The boundary between these nuclei is difficult to establish. Large pale ellipsoidal cells like those of the mesencephalic fifth nucleus, have been seen occasionally in or near the fourth nucleus (36). I have not observed such cells.

Composition and course of the fourth nerve

The fibers of the fourth nerve in the adult human brain collect fanwise from the long column of cells into two fasicles (Fig. 14). These derive not so much from the principle vs. accessory nuclei as from the medial vs. lateral groups of cells. The significance of this segregation is unknown. There is also a small contribution of fibers from cells of the raphe. The most caudal motor cells lie almost at the level of the decussation and their fibers sweep rostrally independently before turning back into the fascicles at various levels. While the cells differ in size the myelinated fibers are rather uniform as they collect. The two fascicles are nearly of the same size and have 500 and 590 fibers respectively.

Where these tracts, extending laterally, meet the border of the central gray matter they turn dorsocaudally. The fiber direction in the embryo is dorsad. The caudal component is the result of differential growth moving the nuclei rostrad and the decussation caudad. On the lateral border of the central gray matter they lie in close contact with both the mesencephalic fifth nucleus and the locus coeruleus and interchange fibers with both nuclei. Both

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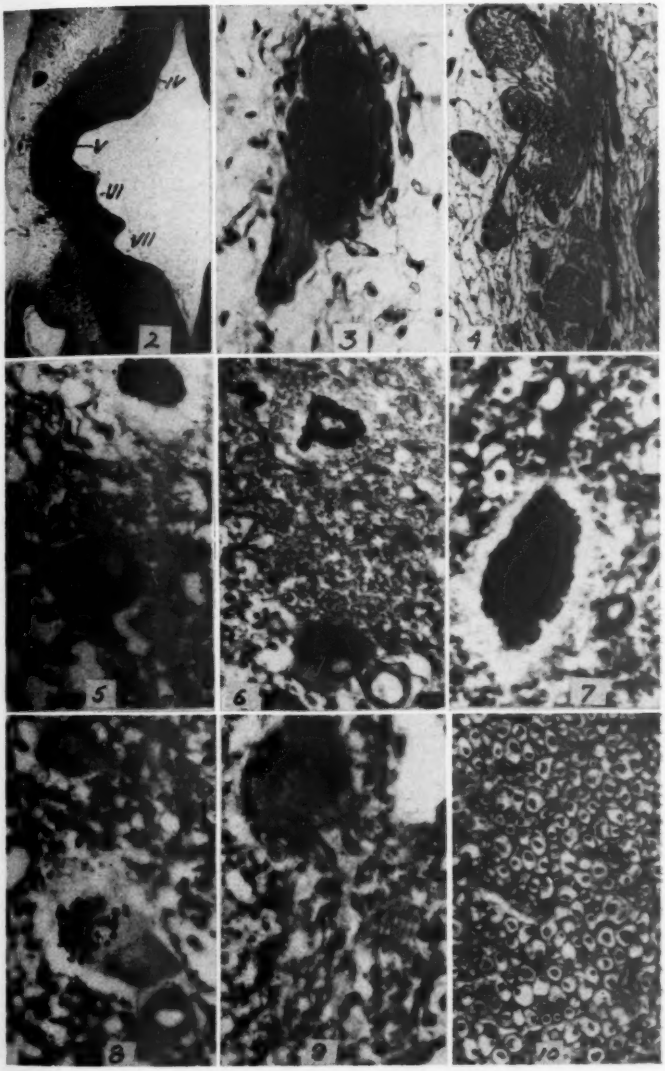
FIG. 2. Hindbrain and neuromeres from a 13 mm. human embryo.
FIG. 3. Fourth nerve and ganglion in a 25 mm. Porpoise embryo.
FIG. 4. Distribution of sympathetic fibers to all parts of ocular muscle nerves and fifth nerve in a 65 mm. human fetus.
FIGS. 5–6–7. Cells of the locus coeruleus and mesencephalic fifth nucleus contrasted in silvered sections of adult human brain.
FIG. 8–9. Intermediate cells with a few reduced silver granules from the same region as figures 5, 6, 7.
FIG. 10. Silver stained detail of the fourth nerve fasicle just before the decussation showing two sizes of myelinated fibers.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nuclei contain large unipolar, bipolar and multipolar cells and though they differ strikingly in general appearance there are cells typically of the mesencephalic fifth type which contain a few granules that will reduce silver like those of the locus coeruleus (Figs. 8, 9, 21, 22, 23). In fact in tissue culture (25) these cells all seem to reduce silver strongly in the peripheral zone, figures 5, 7, 9, show contrasting and intermediate forms. As has been indicated both cell types may have been derived from neural crest equivalents well above the basal plate and spread downward during development. The mesencephalic fifth cells are all lateral to the fourth fasicles (Fig. 14), but pigmented cells lie on both sides or even embedded among them. These cells have a very strong reducing action not found in adjacent cells of other nuclei (Fig. 5), and around each cell is a zone apparently free of any fibers. They are generally bipolar, oriented longitudinally with some lateral processes at right angles in both directions. Russell (40) has made a detailed study of the locus coeruleus nucleus and its connections.

As the fasicles of the fourth nerve rise to the decussation they contact the mesencephalic fifth cells more closely and it is claimed that mesencephalic fifth fibers enter the decussation. In this region however, a large number of small fibers enter the fourth nerve laterally but the source cannot be identified (Fig. 10).

It seems probable that the decussation of fibers of the fourth nerve is complete though the data on chromatolysis (20) and degeneration of boutons (43) are not conclusive. Fibers from the superior oblique muscle have been traced solely to the contralateral nucleus (10). The decussation contains masses of small fibers in addition to fourth nerve fibers. We have also seen a small bundle of fibers from the dorsal border of the brachium conjunc-

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FIG. 11. Fourth nerve detail outside the adult brain. Osmicated.

FIG. 12. A cluster of cells on the fourth nerve at the insertion in a 20 mm. human embryo. Hortega stain.

FIG. 13. The two fascicles of fourth nerve just outside the isthmus. Twenty mm. human embryo. Hortega stain.

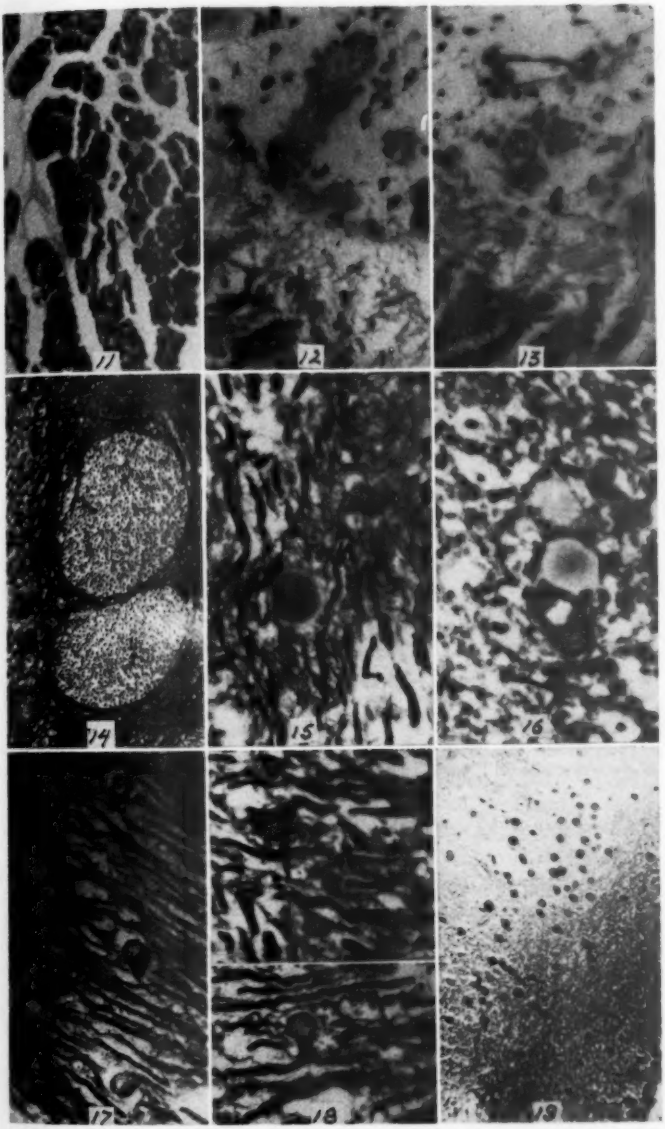
FIG. 14. Section of fourth nerve fascicle in adult human brain contact with the mesencephalic fifth nucleus.

FIG. 15. Silvered amyloid body in the decussation. Adult human brain.

FIG. 16. Two amyloid bodies showing the relationship to a sheath of fine unmyelinated fibers. Silvered adult human brain.

FIGS. 17–18. Cells found in the decussation. Silvered adult human brain.

FIG. 19. Distribution of amyloid bodies in the region of the decussation.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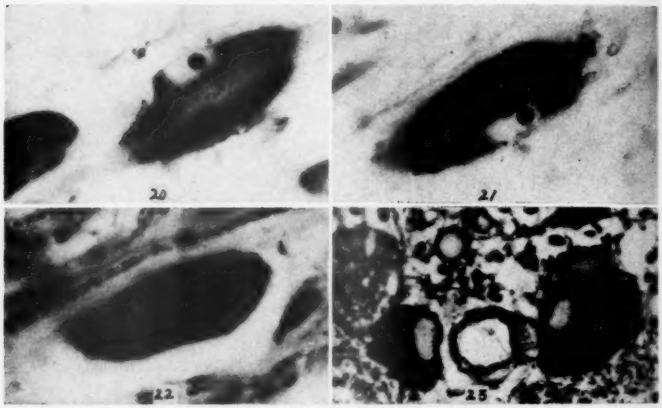
tivum turn sharply and apparently enter the fourth nerve. A few cells have been found in the decussation (Fig. 17, 18). Pearson (37) has noted some in the human fetus. A peculiarity of the isthmus region is the large number of amyloid bodies, both on the surface and in the decussation (Fig. 19). The nature of these bodies is unknown and in silvered preparations they simulate neurons (Figs. 15, 16). They differ from the cells of the locus coeruleus, on whose borders there is complete lack of nerve fibers, by being completely and closely surrounded by myelinated fibers (Fig. 16).



FIGS. 20–21. Cells of locus coeruleus. Kluver stain adult human brain.
FIGS. 22–23. Mesencephalic fifth cells with Kluver stain and with silver adult human brain.

Open mouse brains (Fig. 24) produced by transplacental urethane (44), provide an experimental situation in which the decussation cannot form. All cranial nuclei are developed and nerves can be followed from the base or margin of the open plate but to date no trace of fourth nerve has been seen. Eye muscles are all developed.

The four fascicles interweave in the decussation but remain distinct. They emerge as a pair of rootlets on each side, often as much as 3.5 mm. apart, and continue ventrally along the side of the isthmus for as much as 20 mm. before becoming a common nerve. The marked delay in development of the fourth nerve is

suggestive. It is possible that the spread of the brachium conjunctivum makes it impossible for the fibers to emerge without going over its margin. A lesser problem of the same sort appears in the caudal portion of the third nerve complex. Here the fibers decussate but it is not known whether contralateral cells move across the midline or merely extend to the decussation.

It is clear that fibers have been added to the fourth nerve either in the decussation or immediately prior to it because a fiber count outside the brain was found to be 2182 while the combined counts of the fascicles before decussation was 1090. This is

FIG. 24. Mouse embryo from late gestation. Mother was given intraperitoneal urethane on the seventh day of gestation.

in agreement with Harley's counts (2107–2187). Counts as high as 3400 have been made (23). The larger count from the osmicated peripheral nerve ignored unmyelinated fibers. There is no explanation of the meaning of these extra fibers. The distribution of fiber sizes is clearly bimodal (Fig. 11). Herrick (24) suggests that in Menidia these reach two sizes of muscle fibers. This has been claimed as a general principle (11). The slender nerve follows the tentorial margin which it enters parallel to the sixth nerve. Where these join the third nerve the three are met by

fibers from the superior cervical ganglion. Sympathetic fibers enter all of them and ganglion cells are found along the course of the ophthalmic branch of the fifth nerve (Fig. 4). As these nerves enter the orbit the fourth nerve reaches the medial aspect of the superior oblique muscle while branches of the fifth nerve continue to the trochlea and beyond. Fibers of the fourth nerve also emerge near the trochlea to innervate the tendon (26).

Afferent fibers from ocular muscles

Movements of the eyeballs are accurately registered somewhere in the vicinity of the ocular motor nuclei (16). There is no agreement as to the path followed by sensory fibers. In the older literature (46) the trigeminal nerve was said to mediate all sensibility for the ocular muscles. Abd-el-Malik (1) raised systemic blood pressure by electrical stimulation of the central end of the fourth nerve. By 1902 sensory endings on the tendon of ocular muscle were seen with methylene blue technique (27). In 1910 Chambers, and in 1944 Cooper and Daniel described 40–50 spindles on the proximal third of the superior oblique muscle. Sunderland reported a smaller number. Woolard 1937 pictured spirals of nerve fiber wrapping ordinary contractile fibers, the fibers are said to travel with the fourth nerve in the orbit and then leave it (17). Hines (26) found that all fibers of the fourth nerve peripheral to a cut degenerate. She found two types of end ing on muscle and two on tendon. The stretch reflex disappears when the nerve is cut but other reflexes remain (14). Microelectrodes along the course of the mesencephalic nucleus fail to pick up impulses generated in the eye muscle and yet they reach the general region of the motor nuclei. We again call atention to the very close contact of the fourth nerve cells to both the raphe, medial longitudinal fasciculus, and the supratrochlear nucleus. Then also the nerve after leaving the decussation has more fibers than upon entering it. These fibers enter dorsally but probably not from the mesencephalic fifth nucleus.

Ganglion cells have been seen on a number of motor roots. Pankratz (35) found some on the fourth nerve of the toadfish. Others had seen such cells (38, 47) but it is a question whether these were afferent or sympathetic neurons. Cells on all the ocular nerves of the chick embryo were reported by Rogers (39). I

present two examples. Figure 12 shows a human embryo of 20 mm. at the exit of the fourth nerve from the isthmus. The cells clustered against this root are not large but are very chromatic. Both fascicles of the nerve are seen in silvered section (Fig. 15). The 25 mm. porpoise embryo (Prodelphinius coerulealbus) shows a ganglion 1.5 mm. long containing hundreds of large cells. A single section (of 28) is seen in figure 3. By the 35 mm. stage the cells have dispersed distally.

Contacts with the peripheral branches of the fifth nerve and exchange of fibers have been noted many times. These are particularly clear in early fetal stages. It is possible that the afferent fibers represent several kinds of proprioception and that these are divided between the fourth and fifth nerves. With such abundant guidance the loss or injury of one path might only serve to make the ocular adjustments a little less skillful.

Summary

The fourth cranial nerve of vertebrates shows unique features some of which may be based on the inclusion into and development of the isthmus region. This includes the shift of level and division of the fourth nuclei, locus coeruleus, and mesencephalic fifth. The fourth nerve in the porpoise embryo, among others, possesses a large ganglion for a short period. The nerve in the adult human brain distal to the decussation contains about double the number of fibers it has before decussation. Many of these enter from dorsal levels at or just ventral to the decussation.

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A METHOD FOR THE DETERMINATION OF THE SYSTOLIC BLOOD PRESSURE OF THE UNANESTHETIZED, UNHEATED RAT

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Widespread use of the rat in the study of problems of the circulation has led to the introduction of a variety of procedures for the determination of the blood pressure of this species. The available methods, however, suffer from several inherent deficiencies which may lead to serious errors in the determinations (Noble, 1949). The method to be described permits the attainment of a graphic record on the unanesthetized animal without recourse to heating and gives reproducible results of a high degree of accuracy.

Experimental

Increase in volume of the occluded rat's tail, which occurs when the applied pressure is reduced to the systolic level, is readily reproducible and is a theoretically sound criterion for determining the blood pressure level. This principle, as applied in the method of Williams, Harrison and Grollman (1939) has been utilized with the modification that the plethysmograph surrounding the tail was connected through a Grass Model PT-5 strain gage and a Sanborn preamplifier to a Sanborn Recorder. The mercury manometer, connected to a smaller cuff to occlude blood flow through the tail, is connected by a Model P-23A Pressure Transducer (Statham Instrument Co.) and simultaneously recorded.

Procedure. The rat is inserted into a holder and its tail placed in the plethysmograph as described elsewhere (Williams *et al.*, 1939). The pressure in the plethysmograph is increased to force blood from the distal part of the tail; the cuff is inflated to a pressure in excess of the anticipated systolic blood pressure; and the plethysmographic pressure adjusted to a level at which respiratory movements of low amplitude, as shown in figure I, are recorded. The recorder is now set in motion and the pressure in the

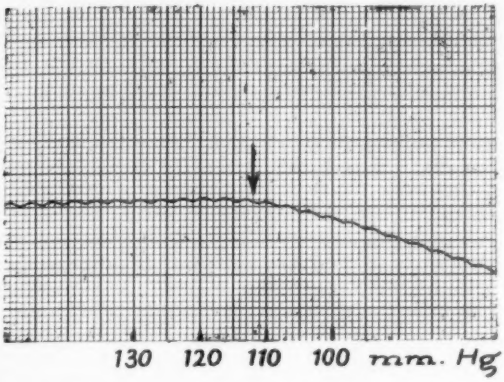

FIG. I. Typical record of a blood pressure curve as obtained on a normal adult rat. The systolic blood pressure is indicated by the arrow as 112 mm. Hg.

occluding cuff gradually reduced. The systolic pressure level is indicated at the point at which the volume curve deviates from the base line.

Results

A typical blood pressure recording is shown in figure I. Reproducibility of the readings is apparent from the series of typical readings (Table 1) obtained over a period of 3 successive days on 10 adult rats, 6 to 12 months of age, weighing 150 to 250 grams each. The results are comparable to those obtained by previously described indirect procedures and are in agreement with those obtained by direct cannulation of the abdominal aorta.

TABLE 1

Daily Blood Pressure Readings of Systolic Blood Pressure of 10 Rats Values Are Expressed in mm. Hg.

Rat No.	1	2	3	4	5	6	7	8	9	10	Average
Day 1	122	130	122	135	135	90	90	95	135	140	119
2	95	140	125	122	125	90	115	75	130	135	115
3	105	150	130	130	122	90	95	90	135	135	118

Discussion

The advantage of the present procedure over previously described methods is the fact that neither anesthesia nor pre-heat-

ing are necessary (as in most currently used methods); the fact that there is no need for applying a cuff to the hindleg (as in the tensometric method) with its inevitable variations leading to erratic results; and the objective nature of the record which obviates subjective influences inherent in methods depending on optical, auditory or similar sensory end-points. The method requires no disturbing manipulation of the animal with its inevitable effects on the blood pressure. Once the apparatus has been assembled the readings may be made by untrained personnel since any aberrant results may be detected from the appearance of the graphic record.

Summary

A method is described for the graphic recording of systolic blood pressure of the rat, which offers advantages over previously available procedures.

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CALLING ATTENTION TO

January, 1958

Preclinical: EP Abraham reviews *Biochemistry of Some Peptide & Steroid Antibiotics* (J Wiley, NY, 57, 96pp $3). RJ Block offers *Amino Acid Handbook* (CC Thomas, Springfield, Ill., 57, 384pp, $10.5). JH Comroe edits symposium on tissue oxygen tension (*Fed Proc* 16/665/57). DL Drabkin edits one on molecular heterogeneity of hemoglobin (*ibid* p 740). RE Forster reviews pulmonary diffusing capacity (*Physiol Rev* 37/391/57). BI surveys origin & secretion of pepsinogen (*ibid* p 475). B Ketterer & Co review relation of pituitary growth hormone to metabolic processes (*Ergeb Physiol* 49/127/57). HA Krebs & PL Kornberg survey energy transformation in living material (*ibid* p 212). F Lynen & K Decker review biological functions of co-enzyme A (*ibid* p327). H Neurath edits symposium on structure & function of protein (*Fed Proc* 16/774/47). EJ Roininger & LA Sapirstein indicate that during digestion there is increased blood flow to all organs (*Science* 126/1176/Dec 6/57). EM Renkin & JR Pappenheimer review permeability of capillary walls (*Ergeb Physiol* 49/59/57). AK Reynolds & LO Randall survey *Morphine & Allied Drugs* (Univ of Toronto Press, Ontario, 57, 393pp, $10). E Shaw & DW Woolley find antiserotonin action in 1-benzyl-2-methyl-5-hydroxytryptamine (*Proc Soc Exp Biol Med* 96/439/57). D Shemin reviews biosynthesis of porphyrin (*Ergeb Physiol* 49/299/57). WM Sperry edits symposium on phosphatids (*Fed Proc* 16/816/57). SE Sulkin & Co show lipotropism of viruses in high rabies virus content of hibernating fat of bats (*Proc Soc Exp Biol Med* 96/461/57). M Theiler finds Na desoxycholate inactivates all arthropor-borne viruses (*ibid* p380). JE Thomas analyses mechanism & regulation of gastric emptying (*Physiol Rev* 37/453/57). N Wald finds increased prothrombin consumption in total body x-radiation (*Proc Soc Exp Biol Med* 96/294/57). JV Warren edits vol 7 *Methods in Medical Research* with sections on chemistry of muscle, human white blood cells, cytology of retina, & hemodynamic methods for heart & lung (Year Book Publishers, Chicago, 58, 250pp, $7.5). RH Wasserman & Co find increased calcium intake helps wash out Sr-90 (*Science* 126/1180/Dec 6/57). *Ciba Symposia:* Edited by GEW Wolstenholme: *Regulation & Mode of Action of Thyroid Hormones; Methodology of the Study of Ageing, & Chemistry & Biology of Purines* (Little Brown, Boston, 1957, 311pp & $8.5; 202pp & $6.5, & 327pp & $9, respectively).

Chlorothiazide: ED Fried & Co find chlorothiazide useful in hypertension (*Med Ann Dist Columbia,* 26/468,516/57). W Hollander & RW Wilkins show its value in arterial hypertension (*Boston Med Quart* 8/67/57). GE Schreiner & HA Bloomer show its chloruretic & natriuretic action (*New Eng J Med* 257/1016/Nov 21/57) & RW Wilkins reviews new drugs for hypertension, recommending "Diuril" Merck (*ibid* p 1026). Drug is 6-Chlorosulfanyl-1,2,4-benzothiadiazine-1, 1-dioxide. Toxicity data not yet clear.

Russian reports: MG Amiragova finds emotional excitement inhibits iodine

uptake by thyroid & stimulates release of thyroid hormones (*Sechenov J Physiol*, English ed, 43/57/57). MA Angarskaya finds sireniotoxin a potent cardiac glucoside, obtained from leaves of aungustifolia (*Pharmacol Toxicol*, English ed, 20/61/57). MA Ignatyeva describes hypotensive & spasmolytic action of triacantine, new alkaloid from *Gleditschia triacanta* (*ibid* p 57). Al Mironenko shows vascular distension in cholesterol atherosclerosis from use of papaverine (*ibid* p 27). VV Zakusov & Co find cardiac glucosides enhance vagal transmission to heart in experimental myocarditis & that ATP blocks this effect (*ibid* p 12).

Clinical: ML Marshall helps arrange *The Physicians Own Library* (CC Thomas, Springfield, Ill., 57, 87pp, $3). P de Nicola describes *Thrombelastography* (CC Thomas, Springfield, Ill., 57, 110pp, $3.5). FA Putnam considers disturbance of protein metabolism in multiple myeloma (*Physiol Rev* 37/512/57). RM Robinson shows that since glucagen reduces gastric motility & acidity it is not cause of Zollinger-Ellison syndrome (*Ann Surg* 14/909/55), gastric ulcers with non-insulin tumors of pancrease (*Proc Soc Exp Biol Med* 96/518/57). H Selye & S Renand show 2-,ethyl-9-chlorocortisol with NaH_2PO_4 & neuromuscular effort can produce myocardial necrosis (*ibid* p 512). JW Wilson discusses *Clinical & Immunologic Aspects of Fungous Diseases* (CC Thomas, Springfield, Ill., 57, 280pp, $6).

General: AG Korol discusses *Soviet Education for Science & Technology* (J Wiley, NY, 57, 513pp, $8.5). EH Mudd & A Krich consider *Man & Wife*: A Source Book of Family Attitudes (Norton, NY, 57, 291pp, $5). G Santillana notes similarities in authoritarian humiliation of unorthodox scientists in 17th & 20th centuries, Galileo & Oppenheimer (*The Reporter*, Dec. 21/57). MB Visscher edits symposium on utilization of recorded knowledge (*Fed Proc* 16/703/57).

February, 1958

Clinical: RP Grant describes *Clinical Electrocardiogrpahy: The Spatial Vector Approach* (McGraw-Hill, NY, 58, 225pp, $7.5). SR Hollan reports anemia follows degeneration of nerves supplying bone marrow (*Acta Physiol Acad Sci Hungaricae* 12/215/57). JP Hubbard edits *The Early Detection & Prevention of Disease* (McGraw-Hill, NY, 58, 350pp, $7.6). Important is KD Keele's *Anatomies of Pain* (CC Thomas, Springfield, Ill., 58, 216pp, $5.5). W. Lochner & E Witzlib edit symposium *Lungen und kleiner Kreislauf* (Lange & Springer, Berlin W35, 57, 169pp, DM 48). LM Morrison finds lecithin most effective cholesterol lowering agent (*Geriatrics* 13/12/58). DE Ryvkina & NE Kuznetsova show histamine causes cardiac reactions to pain, which are blocked by antihistaminics (*Sechenov Physiol J. USSR* 43/230/57). MB Strauss considers *Body Water in Man* (Little-Brown, Boston, 57, 286pp, $7). DC VanDyke & Co find urine of anemic patients high in erythropoietic action (*Proc Soc Exp Biol Med* 95/541/57). PD White & Co describe *Cardiovascular Rehabilitation* (McGraw-Hill, NY, 58, 155pp, $6.5).

Nervous System: OA Briukhanov & PI Lomonos show ionizing radiation reduces conditioned reflex response (*Sech Physiol J USSR* 43/93/57). FJ Fry & Co find ultrasound radiation of lateral geniculate nucleus reversibly stops

potential in visual cortex on light stimulus (*Science* 127/83/Jan 10/58). B Gelber & DD Jensen debate "learning" in paracmecia (*ibid* 126/1340/Dec 27/57). CS Hall & G Lindzey survey *Theories of Personality* (J Wiley, NY, 57, 572pp, $6.5). H Himwich well reviews psychopharmacologic drugs (*Science* 127/59/Jan 10/58). AM Lassch goes over *The Human Brain from Primitive to Modern* (CC Thomas, Springfield, Ill., 57, 256pp, $5). Our S Levine & Co correlate infantile experience with maturation of pituitary-adrenal combine (*Science* 126/1347/Dec 27/57). OR Lindsley finds measure of sleep in relation to operant behavior (*ibid* p 1290, Dec 20/57). VE Maiorchick shows cortical cells in area of pathological focus lose lability under afferent stimuli (*Sech Physiol J USSR* 43/179/57). E Vladmimirova finds cortical excitation is accompanied by increase in ammonia content of cortex (*ibid* p 107). NE Miller describes experiments on motivation (*Science* 126/1271/Dec 20/57).

Preclinical: AA Barber finds ethylene-diamine-tetra-acetate stops lipid peroxide formation in liver & protects succinoxidase in mitochondria from ultraviolet inhibition (*Proc Soc Exp Biol Med* 96/471/57). KH Beyer & Co study electrolyte excretion as influenced by chlorothiazide (*Science* 127/146/Jan 17/58). Jean Brachet offers *Biochemical Cytology* (Academic Press, NY3, 57, 516pp, $8.8). A Brodal analyses *The Reticular Formation of the Brain Stem* (CC Thomas, Springfield, Ill., 58, 160pp, $4). BB Brodie & CAM Hogben survey physico-chemical factors in drug action (*J Pharm Pharmacol* 9/345/57). BM Dzhakson claims central nervous inhibition increases trypsin activity in pancreatic juice (*Sech Physiol J USSR* 43/144/57). A Engstrom & JB Finean review *Biological Ultrastructure* (Academic Press, NY3, 58, 326pp, $8). H Fernandez-Moran & R Brown edit *Submicroscopic Organization & Function of Nerve Cells*, Proc Caracas Inst Neurology, *Suppl* 5 *Exper Cell Res*, 58, 302pp, $10). PI Guliaev relates sleep phases to tidal excitability in EEG patterns (*Sech Physiol J USSR* 43/115/57). B Gunther & E Guerra calculate that men can fly using their own musculature for power (*Acta Physiol Lat Amer* 7/95/57). C Huggins & L Pollice find repeated administration of 3-methylcholanthrene depresses hypophyseal activity (*J Exp Med* 107/13/58). B Lindstrom & R Brown edit *Cytochemical Methods*, Proc Stockholm Inst Med Cell Research, *Suppl 4 Exper Cell Res*, 57, 296pp, $10). QR Murphy edits *Metabolic Aspects of Transport across Cell Membranes* (Univ Wisconsin Press, Madison, 58, 416pp, $7.5). JE Salk & EN Ward note cytotoxic immune reactions in tissue culture (*Science* 126/1338/Dec 27/57), as observed by AA Bogomolets (*Amer Rev Sov Med* 1/101/43) & CM Pomerat & L Anigstein (*Texas Rep Biol Med* 3/122, 404/45). K Thuranszky & Cs Csetri claim reduction in rate of blood flow causes sludging & not viceversa (*Acta Physiol Acad Sci Hungaricae* 12/93/57).

General: A Cornu analyses *The Origins of Marxian Thought* (CC Thomas, Springfield, Ill., 57, 128pp, $3.5). JM Murtagh & S Harris survey prostitution in *Cast the First Stone* (McGraw-Hill, NY, 58, 307pp, $4.5). R Rice gives vivid profile of vivacious musician L Bernstein (*New Yorker*, Jan 11 & 18/58). R Romanell moves *Toward a Critical Naturalism* (Macmillan, NY, 58, 103pp, $3). PB Sears says the inexorable problem of space is right here on

earth (*Science* 127/9/Jan 3/58). RGH Siu offers essay on Western knowledge & Eastern wisdom, *The Tao of Science* (Wiley, NY, 58, 180pp, $4.5).

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Clinical: JA Anderson & Co note high urinary output of 5-hydroxy-indoleacetic acid, diagnostic sign in carcinoid tumors, after eating bananas (*Science* 127/236/Jan 31/58). H Blumberg & Co find bronchodilator action in anticoagulant warfarin (*ibid* p188/Jan 24). WJ Dempster offers *An Introduction to Experimental Surgical Studies* (CC Thomas, Springfield, Ill., 1958, 400pp, $10). MM Draper finds thiamine absorption from gut decreases in aging (*Proc Soc Exp Biol Med* 97/121/58). PH Forsham edits Symposium on *Adrenal Corticoid Therapy* (*Metabolism* 7/1–121/58). WH Gantt compiles *Physiological Bases of Psychiatry* (CC Thomas, Springfield, Ill., 1958, 220pp, $6). JC Krantz & Co find "Fluothane" (trifluoro-bromchloro-ethane) a potent but highly toxic anesthetic (*Anesth* 19/38/58). A Meyer offers *Psychobiology* (CC Thomas, Springfield, Ill., 1958, 272pp, $6.5). JM Moyer & Co find butamide (sulfa carbonic-anhydrase inhibitor) at 150 mgm per day a useful & safe diuretic in cardiac decompensation (*Geriatrics* 13/80/58). IH Page edits *Chemistry of Lipides as Related to Atherosclerosis* (CC Thomas, Springfield, Ill., 1958, 352pp, $10.5). EB Sigg & Co find reserpine antagonizes morphine analgesia while amphetamine potentiates it (*Proc Soc Exp Biol Med* 97/97/58). FJ Smirk gives therapy details for *High Arterial Pressure* (CC Thomas, Springfield, Ill., 1958, 760pp, $15). PT Wertlake & Co note increase in serum cholesterol in mental & emotional stress (*Proc Soc Exp Biol Med* 97/163/58). *General:* JD Adamcs computes *Triumph over Odds* (Duell, Sloan, NY, 1958, 322pp, $6). FR Barta discusses *The Moral Theory of Behavior* (CC Thomas, Springfield, Ill., 1958, 45pp, $2). SC Bacon edits symposium on *Understanding Alcoholism* (*Ann Amer Acad Pol Sec Sci.*, 315/1–146/Jan 58). V Heines translates & annotates Albertus Magnus (1193–1280) *Libellus de Alchimia*, important for technological data (Univ California Press, Berkeley & Los Angeles, 1958, 101pp, $3). B Jaffe finds *Chemistry Creates a New World* (Crowell, NY, 57, 333pp, $4.5). B Karpman analyses *The Hangover a Critical Study of the Psycho-Dynamics of Alcoholism* (CC Thomas, Springfield, Ill., 1958, 560pp, $9.5). A Meares describes *The Medical Interview* (*ibid* 128pp, $3.5). NI Sax & Co give full data on *Dangerous Properties of Industrial Materials* (Reinbold, NY, 57, 1467pp, $22.5). H Wain gives *The Story Behind the Word*, on origin of medical terms with biographical data (*ibid* 352pp $10). *Preclinical:* IL Bennet & LE Cluff review bacterial pyrogens (*Pharmacol. Rev* 9/427/57). WE Connor & JR Carter show soy-bean phosphatide acts as thromboplastin in blood coagulation (*Proc Soc Exp Biol Med* 97/38/58). PF Cranefield & BF Hoffman survey electrophysiology of single cardiac cells (*Physiol Rev* 38/41/58). DC Darrow & S Hellerstein review body water & electrolyte changes (*ibid* p 114). R De Valois & Co find complete color-vision system represented in 2 dorsal layers of lateral geniculate nucleus of monkeys (*Science* 127/238/Jan 31/58). JT Edsall & J Wyman offer Vol I of *Biophysical Chemistry*, ending with biological significance of the properties of matter (Academic Press, NY 3, 1958, 699pp, $14). FF Foldes studies fate of

muscle relaxants in humans (*Acta Anesth Scand* 1/63/57). A Geiger correlates brain metabolism with function (*Physi Rev* 38/1/58). R Hartman & PF Guenther translates P Morawitz's *The Chemistry of Blood Coagulation* (*Ergeb Physiol* 4/307–423/1905) & give glossary index (CC Thomas, Springfield, Ill., 1958, 204pp, $5). W Hild successfully grows neurons in tissue culture (*Ztschr Zellforsch* 47/127/57). JR Hughes reviews post-tetanic potentiation (*Physiol Rev* 38/91/58). K Koizumi & Co note prolonged effects of reticular formation stimulation on reflex action (*Neurol* 8/102/58). J LeBlanc & F Rosenberg find hypothermic action of chlorpromazine, histamine & serotonin reduced by acclimatization (*Proc Soc Exp Biol Med* 96/482/57). J Olds reviews self-stimulation of brain to study local effects of hunger, sex, & drugs, finding reciprocal inhibiting centers in midbrain & rhinencephalon for pleasure & pain (*Science* 127/315/Feb 14/58). CM Pomerat & Co find giant cell formation in human cell strains in tissue culture on exposure to gamma radiation, with high chromosome numbers (*Ztschr Zellforsch* 47/158/57). Reports appear on controlled release of thermonuclear energy (*Nature* 181/217/Jan 25/58). SS Stevens discusses measurement (*Science* 127/383/Feb 21/58). RL Swank & JH Fellman study plasma proteins & fat transport (*Amer J Physiol* 192/311/58). RE Tunbridge edits symposium on *Connective Tissue* (CC Thomas, Springfield, Ill., 1958, 320pp, $11). BL Vallee & JHR Kagi show importance of zinc in metabolism (*Schweiz Med Wchnschr* 88/132/Feb 8/58). G Wald & PK Brown review human rhodopsin (*Science* 127/222/Jan 31/58).

AAAS Science Parliament: For session in Washington, March 15–17, RL Meier proposes a method for anticipating explosive consequences of science, using administrative scientists (*Sat Rev*, Mch 1/58).

C. D. Leake

MECHANISMS OF ARTERIAL BLOOD PRESSURE VARIATIONS

ARTHUR GROLLMAN[a]

The arterial blood pressure is not only readily amenable to measurement but also reflects much concerning the state of the circulation. As a consequence, deviations from normal are readily noted. The significance of such deviations are, however, not always evaluated properly and incorrect inferences are often drawn regarding the significance of a given reading.

Measurement of Arterial Blood Pressure

The approximate maximal and minimal arterial pressures designated as the systolic and diastolic pressure, respectively, are obtained conveniently by the ordinary clinical sphygmomanometer which is adequate for most purposes. However, such determinations are subject to certain technical errors. In the very obese, for example, the use of the ordinary cuff may give values which are often as much as 50 millimeters too high. To obviate this error the cuff should be placed on the forearm and the sounds auscultated over the radial artery. In sclerotic vessels, on the other hand, an erroneously high reading may be obtained if one utilizes the appearance of the sounds to designate the systolic blood pressure level. Palpation of the radial pulse rather than the use of the stethoscope will obviate this source of error.

For exact investigational work and for the determination of the true mean arterial pressure, the curve obtained by an accurate recording device connected to a catheter inserted into the vessel is necessary. In this way one may obtain an accurate mean pressure by planimetric integration. The true mean pressure is not the average of the systolic and diastolic pressures but is influenced by the shape of the pressure curve (2).

Significance of the Arterial Blood Pressure Level

The level of the arterial blood pressure is determined by three variable hemodynamic functions, *viz.*: the cardiac output, the

elasticity of the vascular tree, and the peripheral resistance. Alterations in one or more of these three functions induce the deviations observed physiologically or in disease. Any observed alteration in the blood pressure reflects changes in these three hemodynamic factors. Since these affect the systolic and diastolic pressures differently, one can often deduce the nature of the underlying hemodynamic alteration from the manner in which these two components of the arterial blood pressure are affected.

It is obvious that the level of the blood pressure will vary with changes in cardiac output unless compensatory changes, such as alterations in vascular tone and peripheral resistance, occur simultaneously. Should the latter, for example, not compensate for a decrease in cardiac output, a decline in blood pressure must ensue as in circulatory shock, in myocardial weakness, in loss of extracellular fluid, or in asthenic states.

Of primary importance in determining the level of the arterial blood pressure is the elasticity of the arterial tree. Since the latter loses its elasticity as a result of arteriosclerosis, it is not surprising to find an increased systolic pressure and a widened pulse pressure with the aging process. As long as the elasticity and arteriosclerotic changes are confined to small segments of the vascular tree, the effect on the systemic arterial pressure is insignificant. However, when it involves a large part of the circulatory system, the resultant effect on the level of the blood pressure is significant. A loss of elasticity of the arterial system alone will result primarily in an increased systolic pressure with a normal or decreased diastolic pressure. This condition, common in the aged, and present to some degree in over 50 per cent of individuals over the age of fifty, is designated as systolic hypertension in contrast to the diastolic hypertension which is characteristic of hypertensive cardiovascular disease. Both conditions involve an elevated blood pressure but their significance clinically is quite different.

The elevation in systolic blood pressure observed in generalized arteriosclerosis reflects an inherent change in the vascular tree and may be considered as a compensatory phenomenon. The lowering of such "high blood pressure" is harmful and undesirable since it entails either a reduction in cardiac output or other hemodynamic alterations causing a diminished blood supply to the peripheral tissues.

Systolic hypertension with a normal or low diastolic pressure is also observed in hyperthyroidism, in the presence of an arteriovenous fistula, in aortic insufficiency, etc. In the first mentioned condition, the elevated pressure reflects the decreased peripheral resistance resulting from dilatation of the vascular bed; in the other instances mentioned it compensates for the abnormality of the circulatory system and makes possible the maintenance of a normal blood flow through the tissues.

Lability of the Blood Pressure

The arterial blood pressure is an exceedingly labile function. In order to maintain an adequate blood supply which can be altered in response to the varying needs of the organism, adjustments occur in the cardiac output, tone of the blood vessels, and the distribution of blood in the various parts of the circulatory system. These adjust the blood flow through various areas in accordance with local needs and are usually accompanied by changes in arterial blood pressure. This lability of the blood pressure must be taken into account in evaluating any given reading.

Psychic disturbances are particulary apt to increase the blood pressure and patients in a disturbed state or under stress usually are found to have a blood pressure much in excess of their normal resting values. This may be misinterpreted as reflecting an underlying hypertension. One must accordingly interpret the observed level of blood pressure in terms of the state of the patient and the other physical findings which reflect the presence of disease. The ophthalmoscopic examination and cardiac status are accordingly pertinent in evaluating the significance of an abnormal elevation in blood pressure. It is not uncommon to encounter patients who enter the hospital with a greatly elevated blood pressure which declines to normal levels simply under rest and without specific therapy. The level of the blood pressure *per se* does not always reflect accurately the degree of hypertensive disease present in a given patient (7).

Hypotension

A decline in blood pressure to abnormally low levels is usually an acutely occurring reaction which is secondary to a failure of

the homeostatic mechanisms which maintain the normal blood pressure. Such a drop may result from cardiogenic or peripheral mechanisms and is a feature of the advanced stage of circulatory shock.

Postural hypotension which produces dizziness or faintness when the patient changes to the erect position occurs as a result of the failure of the normal compensatory mechanisms which overcome the effect of gravity. Pressure on the carotid sinus, intermittent heart block, and ventricular asystole often produce abrupt falls in arterial blood pressure which may cause fainting. Hyperventilation may also induce a similar reaction, particularly in patients with heart disease and left ventricular failure who are predisposed to syncope. Exertion also may precipitate syncope, especially in patients with aortic stenosis or aortic insufficiency. It is the failure of the autonomic mechanisms which mediate the adjustments in arteriolar tone and the response to changes in posture which are responsible for the fainting and dizziness observed in elderly persons suffering from generalized arteriosclerosis.

Patients whose blood pressure is at a lower level than the average observed in others of their age group are often considered as suffering from "hypotension" but this concept in erroneous. In the absence of generalized arteriosclerosis or hypertensive disease, the blood pressure would be anticipated to remain at the same level in the aged as in the young individual. To designate such individuals as suffering from "hypotension" is thus unjustified. Patients who are apathetic or mentally depressed often manifest a blood pressure lower than the average for their age, but this lowering is a reflection rather than a cause of the symptoms which they manifest. Addison's disease, pituitary insufficiency and other disturbances of a chronic nature also result in a lowering of arterial blood pressure, which is secondary to a loss of extracellular fluid volume.

Alterations of Blood Pressure with Age

The loss of elasticity of the blood vessels with increasing age results, as already indicated, in the development of systolic hypertension. This alteration in the blood vessels is evident during early youth, but does not appear to any significant degree until the later years and may be absent even in aged individuals.

Although apparently in part an inevitable consequence of the aging of protoplasm, it may be accelerated by other factors such as certain diseases and possibly by dietary and other environmental factors. However, there is no true parallelism between the vascular changes and senility since many symptoms of the aging process are apparently independent of alterations in the blood vessels.

Although the blood pressure generally rises from childhood to old age, the systolic pressure does not normally continue to rise after about the age of 70 nor does the diastolic pressure do so after the age of 65. In general, the systolic pressure in women over 50 is higher than in men, but after the age of 90 this difference becomes slight. In 95 per cent of persons between the ages of 65 and 100 the systolic pressure ranges from 100 to 212 and the diastolic level from 55 to 112; that is, from normal to hypertensive levels (5). The fact that these abnormally high levels are present in the majority of individuals above the age of 50 reflects the fact that abnormality of the vessels with loss of their elasticity as a result of generalized arteriosclerosis is a common pathological finding in aging individuals. The slight decline in diastolic pressure and constancy of the systolic pressure after the age of 65 is a result of the selective morbidity and mortality associated with an elevated blood pressure, since only those with the lower values survive to the higher age groups (1).

Hypertensive Cardiovascular Disease

The most important (although not the most common) cause of an elevation in blood pressure is a disease process designated as hypertensive cardiovascular disease. This condition is a specific clinical entity of great practical importance.

Although for a long time considered as an inevitable consequence of the aging process, hypertensive cardiovascular disease is now looked upon as a specific disease. The great mass of experimental data accumulated during recent years has elucidated our concepts of this disorder. It has been demonstrated, for example, that the fundamental hemodynamic disturbance in the early stages of the disorder is an increase in peripheral resistance. This is accompanied by a normal cardiac output so that despite the elevation in blood pressure the vascularization of the tissues remains at a normal level.

The characteristic and essential change in the arterial blood pressure in hypertensive disease is an elevation in diastolic pressure, which differentiates it from the various forms of systolic hypertension discussed above. This elevation in diastolic pressure is, of course, accompanied by an increase in systolic pressure with an increased pulse pressure which is requisite for maintaining a normal blood flow and normal cardiac output. It is only after the condition has existed for some time that secondary arteriolosclerosis, the characteristic pathological finding in hypertensive cardiovascular disease, makes itself manifest. The latter is the pathological finding which, with cardiac hypertrophy, are the pathological hallmarks of the disease and allow the diagnosis to be made even if the patient is observed first at autopsy without any knowledge of the pre-existing level of his blood pressure.

Hypertensive disease is characterized pathologically by the development of medial hyperplastic sclerosis in which there is marked thickening of the medial layers as opposed to the intimal sclerosis designated as atherosclerosis. This form of arteriosclerosis is specific for hypertensive cardiovascular disease in its so-called benign phase. In the malignant phase of the disorder necrosis of the blood vessels occurs with the characteristic picture evident in the eye grounds by hemorrhage and exudate seen through the ophthalmoscope.

Although some have expressed doubt as to the existence of hypertensive cardiovascular disease as a distinct entity, there is no reason to do so if one defines properly the condition and excludes other clinical states in which there is an alteration in the blood pressure. Its well-known clinical course, its hemodynamic and pathological features leave no reason to doubt its authenticity as a clinical entity (3). Moreover, it is possible to produce the disease in the experimental animal by various manipulations, all of which have in common the fact that they affect the kidney. Thus, interfering with the blood supply to the kidney by means of a clamp on the renal artery with extirpation of the contralateral organ, reducing the effective mass of functional tissue, dietary deficiencies which induce alterations in the kidney, poisonings which affect the parenchyma, the application of a figure-of-eight ligature around the kidney and removing the contralateral kidney, etc., induce the disease with the same hemodynamic, clinical and pathological features as characterize the

disease in man. A crucial experiment is the demonstration that removal of all renal tissue from the animal by bilateral nephrectomy results in the features of the so-called malignant phase of hypertensive disease.

The above-described experiments would indicate that hypertensive disease is of renal origin despite the fact that it may be induced by various mechanisms. One can easily explain these as acting through the kidney, either by affecting its parenchymal tissue, its blood supply or functional activity by other means.

Mechanisms Involved in the Pathogenesis of Hypertension

As already indicated, the blood pressure is a labile hemodynamic function. Attempts have been made to attribute the elevation in blood pressure to the impact of factors which normally raise it. The fact, for example, that the blood pressure rises following stimulation of the sympathetic nervous system suggested early that repeated nervous stimuli might ultimately lead to hypertension. However, attempts to induce sustained hypertension in this manner have failed. The alleged tendency of hypertension to develop in those subjected to frequent psychic disturbances or to the stresses of modern civilized life is also not supported by any available data. The concept that hypertension is most prevalent in persons with repressed hostility or manifesting other specific psychologic characteristics is also without basis.

A labile and intermittent elevation in blood pressure has been designated as neurogenic hypertension. Such a condition may be induced experimentally by cutting the moderator nerves. However, the hemodynamic, pathologic, and clinical features of this condition differ radically from that of hypertensive cardiovascular disease and hence the two should not be confused. In the experimental laboratory animal, which like man responds to stress by an elevation in blood pressure, the continuous application of such stresses for long periods fails to induce chronic hypertension. There is thus no justification for imputing to the nervous system a role in the pathogenesis of hypertension, although the level of the blood pressure in the hypertensive patient remains under nervous control and can be altered by neurogenic influences.

The endocrine organs, particularly the adrenal cortex, also influence the level of the blood pressure and have therefore been

implicated in the pathogenesis of hypertension. However, it has been demonstrated that the adrenals play a permissive role only in the maintenance of blood pressure. Adrenal cortical insufficiency by depleting the extracellular fluid volume, as well as by its general debilitating effects, induces a decline in blood pressure in both the normal and hypertensive. However, the fact that malignant hypertension can be induced by nephrectomy in the absence of the adrenals indicates that these glands play no primary role in the pathogenesis of hypertension (3).

Alterations in salt and water metabolism also affect the blood pressure. The long continued administration of salt without corresponding increases in potassium of the diet induces chronic hypertension (6). Drastic sodium restriction, on the other hand, in animals and in man tends to lower the blood pressure. However, there is no reason to believe that these observed changes reflect any direct relation between the two processes. Since hypertension is a systemic disorder, one might anticipate finding many metabolic, morphologic, and functional alterations which are only secondary to some more basic disturbance responsible for the development of the disease.

Of special importance in interpreting the probable pathogenesis of hypertension in man is the role of heredity. The available evidence would indicate that this plays an important and probably the primary role in the transmission of so-called hypertension. The determination of the relation of such pathogenetically distinct disorders as coronary heart disease, cerebral vascular accidents and other disorders of the cardiovascular system, and the difficulties of obtaining a reliable family history, have rendered an exact analysis of the hereditary factors in hypertensive disease difficult. However, it would appear that essential hypertension is a dominant hereditary trait with a rate of expression in excess of 90 per cent and that a positive family history suggests with a probability of about 6 to 1 that a given patient is suffering from this disorder (7).

Role of the Kidney in the Pathogenesis of Hypertension

The available experimental data indicates that hypertensive cardiovascular disease is of renal origin. However, the manner in which dysfunction of the kidney induces hypertension is a

matter that has not been established with certainty. Earlier workers conceived of the idea that the elevation in blood pressure was a result of the liberation by injured renal tissue of a pressor agent. This concept originated during the last century with the experiments of Tigerstedt and Von Bergmann, who named the active principle renin. It was subsequently demonstrated that the active pressor agent, which has been designated as angiotonin, hypertensin, or angiotensin, is a product of the reaction of renin with renin substrate to form a peptide which has been synthesized.

The presently available evidence would indicate that this pressor agent plays a part in the induction of hypertension only in a few instances, particularly following infarction of the kidney and acute anoxia. Under these circumstances the removal of the affected kidney results in amelioration of the hypertension. On the other hand, in most instances of so-called chronic benign hypertension there is no evidence that it is the liberation and presence of such a pressor agent that is responsible for the manifestations of the disease.

An alternative hypothesis which has been presented to explain the renal origin of hypertension postulates that the kidney produces an essential material of a humoral nature which is necessary for the maintenance of the normotensive state. Deficiency of this agent, according to this theory, may be induced by many conditions, all of which act by interfering with the normal function of the kidney concerned in the maintenance of the normotensive state. This renal deficiency results in the disorder designated as hypertensive cardiovascular disease. The acceptance of either theory would give one a starting point for the logical treatment of the disease which at present remains irrational, since it depends upon nonspecific measures such as interference with the nerve supply to the blood vessels or the use of drugs which depress the tone of the blood vessels and thus induce a drop in blood pressure without affecting the fundamental disturbance in the kidney.

Conclusion

The level of the arterial blood pressure is a labile hemodynamic function which is altered in many physiological as well as pathological states. Since an elevation in arterial blood pressure may

be due to a variety of causes, a failure to differentiate these and the designation of all increases above normal as "hypertensive" has led to considerable confusion. The great majority of elevations encountered clinically involve primarily the systolic pressure and are a result of arteriosclerosis. The disease, designated as hypertensive cardiovascular disease, should be considered as a specific clinical entity. Recent studies have shown this disorder to be renal in origin and have indicated ways for the amelioration of the condition. Control of the disease is of the greatest practical importance since it would obviate the cerebral vascular accidents and the cardiac and renal failure which are the common fatal complications of this disorder.

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INCIDENCE OF VARIOUS TYPES OF CARDIOVASCULAR DISEASE BY AGE GROUP IN THE MALE AND IN THE FEMALE[a]

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Cardiovascular diseases consistently cause approximately one-half of all deaths in the United States each year. Arteriosclerosis and hypertension together account for more than 90 per cent of all of these deaths (30). The causes of these diseases are unknown, but a study of their incidence might help to point out certain important factors involved in their development. This paper is concerned primarily with arteriosclerosis and hypertension, but the incidence of congenital heart disease, rheumatic heart disease, and atrial fibrillation will be discussed briefly with respect to the inherent nature of the cardiovascular system in the male and in the female.

ATHEROSCLEROSIS AND CORONARY HEART DISEASE

Age and Sex Incidence

Atherosclerosis, the type of arteriosclerosis characterized by an intimal accumulation of amorphous lipid comprises at least 85 per cent of all arteriosclerotic lesions (22). Atherosclerosis involves all arteries, but the involvement of the coronary tree is of particular interest because of the differences in incidence with respect to age group and sex.

Many authors have emphasized the difference between the occurrence of coronary heart disease in males and in females. However, considerable disagreement exists as to the incidence of this disorder. Several criteria may be used for defining coronary artery disease including clinical myocardial infarction or angina pectoris, the degree of coronary atherosclerosis present at autopsy, or heart failure and death attributable to coronary disease. The degree of atherosclerosis found at autopsy cannot always be directly related to clinical coronary disease (35). Also, the incidence of coronary heart disease is difficult to establish because groups representing an adequate distribution of the population are difficult to obtain.

TABLE 1

Comparison of Incidence of Clinical Coronary Artery Disease According to Sex

Authors	Reference Number	Total Cases	Per cent Males	Per cent Females	Male:Female Ratio
Bland and White	7	200	84	16	5:1
Parkinson and Bedford	33	83	86	14	7:1
Schwab and Schulze	40	335	79	21	4:1
Levy and Boas	24	1,228	84	16	4.9:1
Levine and Brown	23	145	70	30	3:1
Mintz and Katz	29	500	68.5	31.5	2.2:1
Parkinson and Bedford	34	187	91	9	9:1
Conner and Holt	15	287	85	15	7:1
Bean	17	300	71	30	3:1
White	18	200	94	4	24:1*
				Average	5:1

* Incidence below the age of 40 years and not included in the average.

TABLE 2

Comparison of Incidence of Autopsy Proven Coronary Disease According to Sex

Authors	Reference Number	Total Cases	Per cent Males	Per cent Females	Male:Female Ratio
Zinn and Cosby	48	700	68	32	3:1*
Clawson	13	1,215	71	29	2.5:1†
Clawson and Bell	14	2,829	63	37	1.7:1*
Willis, Smith, and Sprague	49	1,431	75	25	3:1†
				Average	2.6:1

* Record of deaths resulting from coronary disease.
† Record of presence of coronary atherosclerosis of moderate to severe grade.

Tables 1 and 2 record the incidence of clinical coronary artery disease and of autospy-proven coronary disease with respect to sex as reported in the literature. Most of the clinical studies have been on selected small groups of patients in which male-female ratios were mentioned as incidental findings with no attempt at representative sampling of the population. The discrepancies between these clinical findings and autopsy reports of coronary disease are immediately apparent.

A review of 1321 consecutive case reports of myocardial infarction based on combined electrocardiographic and clinical evidence was made for this paper. These cases include every myocardial infarction on record in two large general hospitals.[d] The total number of males and of females in the population served by these general hospitals are approximately equal. Of

this relatively large group, 952 (72 per cent) were males and 369 (28 per cent) were females which gives a 2.6:1 ratio of males to females. This ratio corresponds with the average of the ratios of autopsy-proven coronary artery disease. The ratio in this study is considerably lower than the average of the previously reported studies on clinical coronary disease (5:1). Since the study made for this paper includes a large number of patients representing all age groups, it is possibly a more accurate estimate of the incidence than some of the clinical studies on smaller selected groups.

Figures I and II show the incidence of these 1321 cases of myocardial infarction according to sex and age. Figure III demonstrates, on a cumulative percent basis, the difference in onset

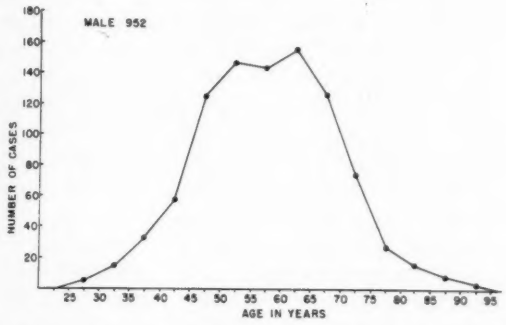

FIG. I. Incidence of myocardial infarctions in males according to age. A study of 952 cases.

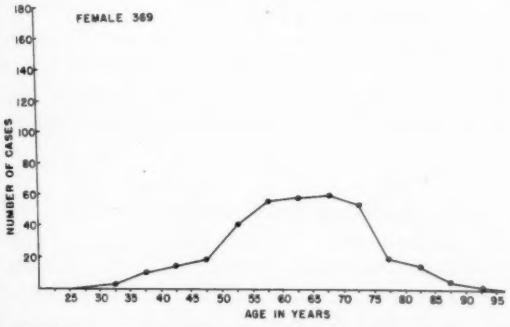

FIG. II. Incidence of myocardial infarctions in females according to age. A study of 369 cases.

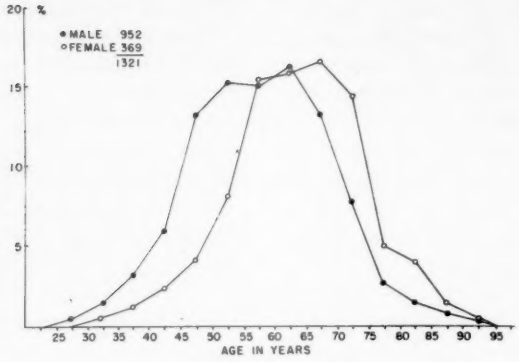

FIG. III. Incidence of myocardial infarctions in males and in females on a cumulative per cent basis.

between males and females. The incidence of myocardial infarctions in males takes a sharp rise rather early in life and reaches a plateau before 55 years. The abrupt fall in the curve of incidence in males after 63 years results from a decrease in the male population after that age and perhaps from a decline in the development of atherosclerosis in the later years (45). The incidence in the females takes a much slower rise until after the age of 45 years when an abrupt increase occurs, and the height of the disease is reached several years after that of the males. According to this data, the male-female ratio below the age of 40 years is about 6:1 as compared to a 24:1 ratio of myocardial infarctions as reported by Gertler, Garn, and White (19). After 55 years the ratio is less than 2:1, and after 65 years the occurrence is about equal for both sexes. These data suggest that certain factors are either retarding the development of coronary disease in the female or accelerating the process in the male.

Contributory Factors

Many factors including diet, blood lipids, heredity, body build, personality, hypertension, endocrine imbalance, and associated metabolic diseases have been implicated in the development of coronary disease. Several of these factors will be considered to determine whether or not they explain the difference between the two sexes. The case of 78 males and 49 females below the age

of 55 years with myocardial infarctions have been carefully reviewed to supplement the literature findings on factors associated with the development of coronary heart disease. These groups were chosen because of the marked difference in incidence of this disease before this age.

Endocrine Factors

Many factors have suggested that female sex hormones may be important in preventing coronary atherosclerosis and that after the menopause the female is no longer protected (22, 19). Nineteen (39 per cent) of the 49 females below 55 years with coronary disease studied for this paper had either their menopause before 45 years of age or absence of ovarian function resulting from surgical removal or irradiation. Thirty-four (70 per cent) of the 49 were between the ages of 50 and 55 years, when most women have passed the menopause.

Experimentally, Stamler, Picks, and Katz (41) have demonstrated that sexually mature chickens exhibit a significant sex difference in susceptibility to cholesterol-induced coronary atherosclerosis. The same group (36) has reported that estrogen treatment of cholesterol-fed male chicks inhibits coronary atherogenesis.

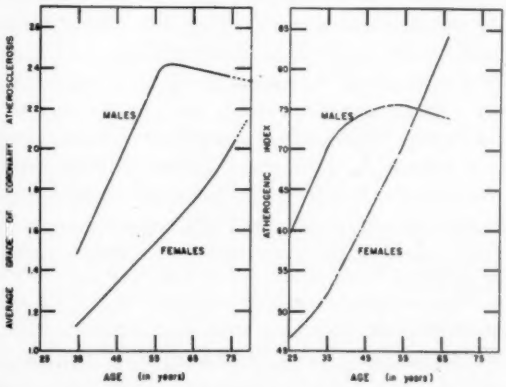

FIG. IV. Comparison of the average grade of coronary Atherosclerosis and Atherogenic index of Gofman with age. The lesions are graded 1–4 with increasing severity. (From Gofman *et al.*, *Mod. Med.*, 21: 119, 1953).

Serum Lipids

Considerable evidence suggests a relationship between the levels of blood lipids and the incidence of coronary artery disease. The importance of serum lipoproteins in this relationship has been emphasized by Gofman and his associates (20). Figure IV shows a comparison of the average degree of coronary atherosclerosis and the Atherogenic Index of Gofman for the two sexes at various age groups (21). The Atherogenic Index is derived from serum lipoprotein values as related statistically to the occurrence of coronary disease. Serum lipoprotein levels appear to parallel the development of atherosclerosis in both males and females. Low serum lipoprotein levels may partly explain the delayed development of coronary disease in the female. Several reports indicate that serum lipoproteins may be decreased in patients with coronary disease by the administration of female sex hormones (3).

Hypertension

The series of 78 males and 49 females below 55 years with myocardial infarction studied for this paper were reviewed for a history of hypertension. Thirty-three (67 per cent) of the 49 females and twenty-five (32 per cent) of the 78 males had a history of moderate to severe hypertension preceding the myocardial infarction. These results are in good agreement with a study of hypertension and coronary occlusion in a similar group which was recently reported by Master (26). He found that 70 per cent of females and 26 per cent of males below 55 years with myocardial infarction had a history of hypertension. Hypertension would appear to be an important factor in the development of coronary disease in the female. In both the female and the male the incidence of hypertension in the coronary group exceeds that of the normal population, and this condition must certainly accelerate the atherosclerotic process even though it may not be the initiating cause.

Other Metabolic Diseases

Certain diseases are associated with a high incidence of atherosclerosis and coronary disease. In these diseases, the incidence in males and in females appears to be about equal. Two important examples are familial xanthomatosis (46) and diabetes mellitus

(14) which are accompanied by high blood lipid levels for both sexes.

Discussion

The above findings indicate that atherosclerosis resulting in coronary occlusion could be the end-product of several different processes. Theoretically, the role of all the clinical findings related to the atherosclerotic process may be explained through four basic factors including time for development, the state of the arterial wall, the level of serum lipids, and the level of blood pressure. These four factors appear to be additive to some degree, and the extent to which each contributes will determine the final degree of atherosclerosis present.

The findings on the incidence of coronary disease may help to evaluate the importance of each of the basic factors involved in atherogenesis. The increased incidence with age may be explained by the time factor, the increase of blood lipoproteins and blood pressure with age, and the changes in the arterial wall on aging. The difference in coronary atherogenesis in the male and in the female may result either from differences in the blood lipids or from a difference in the state of the coronary arteries of the two sexes. Dock (17) has reported that the coronary arteries of males have thicker intimas at birth than females which might establish a background for the more rapid development of atherosclerosis in the male.

Elevations in serum lipids as in diabetes or in familial xanthomatosis or severe hyptertension appear to remove the sex difference usually seen in coronary artery disease which supports the role of serum lipids and high blood pressure as accelerating factors in atherosclerosis.

Hypertension and Hypertensive Cardiac Disease

The importance of hypertension as a clinical condition is well recognized, and along with arteriosclerotic cardiovascular disease, hyptertension-induced diseases constitute one of the greatest causes of death in the United States. Because of the chronic nature of most cases, the incidence of hypertension is even more difficult to determine than that of coronary heart disease. Besides errors resulting from inadequate sampling of the population and inac-

curate recordings, definitions of hypertension differ with almost all authors.

By far the most common type of high blood pressure is essential hypertension constituting 80-85 per cent of all hypertension. The chronically elevated blood pressure of essential hypertension produces multiple deleterious effects on an individual by damaging especially the heart, kidneys, and brain (5).

Although the damage produced by long-standing elevated blood pressure is recognized, the level of blood pressure which should be classified as hypertension is open to question. The definition of hypertension used is of utmost importance in consideration of the incidence according to age group and sex. The important consideration in defining hypertension is the question of what blood pressure will produce deleterious effects on the various organ systems if maintained for a considerable period of time. This question is also of extreme importance in the prognosis and treatment of hypertension, but much disagreement exists regarding the answer to this problem.

The presently accepted blood presure limits which were derived from the early work of Burton (11), Cook (16) and others have become firmly established by the insurance companies. However, many authors (8, 12, 26) have demonstrated that patients classified as hypertensive may show no clinical symptoms for many years. Master and associates (26, 27) have offered new definitions of normal blood pressure and hypertension based on their study of 74,000 working men and women between the age of 16 and 65 years. Table 3 shows the normal blood pressure and lower limits of hypertension proposed by these authors. Other authors (38) have emphasized the importance of maintaining the previously set lower limits of hypertension from a prognostic standpoint.

Figure V shows the incidence of hypertension in the later decades of life as reported by several authors. The results vary considerably depending upon the lower limits of hypertension used, the sex studied, and the group selected. These findings indicate that accurate incidences according to age group have not been established. According to Master's new definition of hypertension, the incidence of essential hypertension is much lower than those previously reported being about 3 per cent in the

TABLE 3

Normal Range and Limits of Hypertension, Newly Defined by Master
(From Master, A. M. Circulation 8:170, 1953)

Age	Systolic Blood Pressure				Age	Diastolic Blood Pressure			
	Normal Range		Hypertension Lower Limit			Normal Range		Hypertension Lower Limit	
	Male	Female	Male	Female		Male	Female	Male	Female
16	105–135	100–130	145	140	16	60–86	60–85	90	90
17	105–135	100–130	145	140	17	60–86	60–85	90	90
18	105–135	100–130	145	140	18	60–86	60–85	90	90
19	105–140	100–130	150	140	19	60–88	68–85	95	90
20–24	105–140	100–130	150	140	20–24	62–88	60–85	95	90
25–29	108–140	102–130	150	140	25–29	65–90	60–86	96	92
30–34	110–145	102–135	155	145	30–34	68–92	60–88	98	95
35–39	110–145	105–140	160	150	35–39	68–92	65–90	100	98
40–44	110–150	105–150	165	165	40–44	70–94	65–92	100	100
45–49	110–155	105–155	170	175	45–49	70–96	65–96	104	105
50–54	115–160	110–165	175	180	50–54	70–98	70–100	106	108
55–59	115–165	110–170	180	185	55–59	70–98	70–100	108	108
60–64	115–170	115–175	190	190	60–64	70–100	70–100	110	110

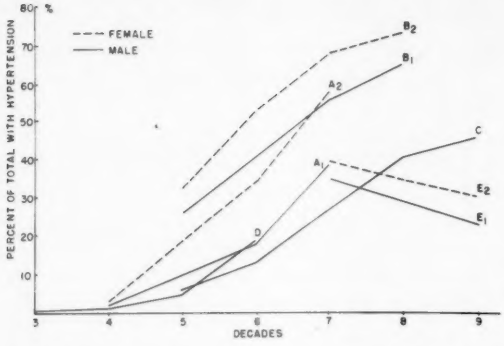

FIG. V. Incidence of hypertension by age. Reports of various authors.

Legend

	Author	Reference	No. Examined	Lower Limit of Hypertension	Sex
A_1	Life Extension	Proc. Life Ext. Exam 1:66, 1939	6,975	150/100	M
A_2	Life Extension	Proc. Life Ext. Exam 1:66, 1939	3,025	150/100	F
B_1	Master *et al.*	Am. Med. A. 121:1251, 1943	14,849	150/90	M
B_2	Master *et al.*	Am. Med. A. 121:1251, 1943	14,849	150/90	F
C	Russek *et al.*	Am. Heart J. 32:468, 1946	5,331	150/95	M
D	Levy *et al.*	J. Am. Med. A. 135:77, 1947	22,741	150/90	M
E_1	Monroe	Disease of Old Age Harvard U. Press, 1951	4,088	/90	M
E_2	Monroe	Disease of Old Age Harvard U. Press, 1951	3,913	/90	F

TABLE 4

Incidence of Hypertension in Males and in Females

Author	Reference Number	Number of Cases	Hypertension Male	Hypertension Female	Age Group	Lower Limit of Hypertension
Boynton and Todd	10	75,218	7.3	1.4	18–25	140/90
		75,218	6.1	2.5	18–25	/90
Alvarez *et al.*	2	15,934	20.0	2.7	25	140/
Symonds	42	162,356	5.7	3.5	16	140/
Bowers	9	461	10.0	13.0	40–70	150/90
Master *et al.*	28	14,849	40.9	50.7	40–90	150/90
Gager	18	2,000	12.6	16.1	All ages	140/90
Wetherby	43	5,540	15.1	21.6	All ages	150/
Schwab and Schulze	40	10,188	3.4	10.6	All ages	150/95

fourth decade, 6 per cent in the fifth decade, and 10 per cent in the sixth.

The reported incidences of hypertension according to sex are presented in Table 4 and Figure V. Before 25 years of age the incidence in males exceeds that in females (10, 2, 42), but after the age of 40 years, according to most authors, the females exceed the males as to hypertension. The cause for this difference in sex incidence has not been explained.

Although the incidence of hypertension is greater in females, the sequelae affect males more commonly. The male is more likely to develop the malignant phase of hypertension (32). Blackford and Wildenson (6) found that the mortality rate after ten years of hypertension was twice as high in men. This increased mortality in men may be intimately associated with the accelerating effect of hypertension on coronary atherosclerosis which is more extensive in males.

Other Cardiovascular Diseases

Congenital Heart Disease

With the recent advances in the field of cardiovascular surgery, and with our increasing knowledge of some of the underlying causes of congenital heart disease, a renewed interest in the incidence and diagnosis of congenital defects of the heart has appeared (46). Congenital anomalies may be inherited or acquired during fetal life. The latter type is of special interest because of the possibility of prevention.

According to Packford and associates (31) the incidence of congenital heart disease in school children is probably between 0.25 and 0.50 per cent. McMahon, McKeown, and Record (25) reported an incidence of 0.32 per cent in 194,418 births which is in agreement with the studies on school children.

A review of 1028 cases of congenital heart disease from a children's hospital[e] was made for this paper. The majority of the diagnoses were confirmed by autopsy, cardiac catheterization, or cardiac surgery. Abbott (1) has published a well known study of 1000 cases of congenital heart disease with which our 1028 cases have been compared as to sex incidence (Table 5). A remarkable agreement exists between the two in most of the defects. Coarctation of the aorta, anomalies of the semilunar cusps, and trans-

TABLE 5

Incidence of Various Congenital Cardiovascular Anomalies According to Sex

Classification of Defect	Abbott's Series			Present Series		
	Male	Female	Total	Male	Female	Total
Simple Dextrocardia	10	6	16	15	7	22
Dextrocardia with Situs Inversus	3	4	7	0	3	3
Defects of Intraauricular Septum	32	39	41	14	22	36
Patient Forman Ovale	15	24	39	5	9	14
Defects of Intraventricular Septum	29	29	58	100	81	181
Complete Defect of Cardiac Septa	17	8	25	12	18	30
Persistant Truncus Arteriosus	11	7	18	12	7	19
Transposition of Arterial Trunks	42	18	60	42	24	66
Pulmonary Atresia	17	10	27	14	17	31
Aortic Stenosis and Atresia	20	13	33	..	..	..
Eisenminger Complex	..	..	..	7	7	14
Anomalies of Semilunar Cusps	34	5	29	..	..	..
Tricuspid Stenosis	1	2	3	11	21	32
Mitral Stenosis	3	3	6	9	9	18
Patent Ductus	36	61	97	41	85	126
Coarctation of Aorta						
Infantile type	4	2	6	26	12	38
Adult type	53	15	68	9	4	13
Tetrology of Fallot	..	..	..	59	56	115

position of the arterial trunks predominate in the male while patent ductus arteriosus and atrial defects are more common in the female.

Almost all authors agree that the total incidence of congenital cardiovascular anomalies is about equal for both sexes (25, 46, 1). The number of males to females in our 1028 cases was 521:507. Although this equal ratio suggests that neither sex has an inherently inferior cardiovascular system, Dock (17) reports that thicker coronary arteries exist in male infants which may partly explain the greater incidence of coronary artery disease in this sex.

Rheumatic Heart Disease

The incidence of rheumatic heart disease is about 1 to 2 per cent in school children where rheumatic fever is prevalent (37). Of ninety-one cases of rheumatic fever reviewed for this paper 59 (65 per cent) were females and 32 (35 per cent) were males which is in accord with literature findings (47).

Atrial Fibrillation

The incidence of atrial fibrillation is consistently higher in males than in females despite the more common occurrence of rheumatic heart disease in females (46). Sixty-two (67 per cent) of 93 cases of atrial fibrillation studied for this paper were males and 31 (33 per cent) were females.

Summary

This paper has attempted to analyze the role of age and sex incidence in our understanding of causes of cardiovascular diseases. Sex differences in incidence of coronary artery disease suggest certain endocrine factors in development of atherosclerosis. The role of serum lipoproteins in atherosclerosis is supported by their different values in males and in females. The accelerating effect of hypertension on coronary atherogenesis, especially in females, has been emphasized. Females with lack of ovarian function or with elevated serum lipids associated with diabetes show an incidence of coronary disease similar to males.

A study of the incidence of essential hypertension contributes less to an understanding of its etiology than with coronary disease, but the sudden onset and rapid increase of the disease after the fourth decade may be of significance.

Equal male-female ratios for combined congenital heart defects suggest that in neither sex is the circulatory system inferior to the other. The obvious difference between males and females with respect to coronary artery disease has led many to believe that the male possesses a weaker cardiovascular system than the female. The unexplained predominance of atrial fibrillation in the male might support such a concept, but the more common occurrence of rheumatic heart disease in the female would refute such an idea. At the present time, no strong evidence indicates the existence of any difference between the cardiovascular systems of either sex if external factors affecting them are excluded.

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EFFECT OF RAT PITUITARY ANTIBODIES ON THE INJECTED RAT[a]

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For the past several years this group has been engaged in the study of anti-organ sera produced by rabbits which were immunized against various rat tissues. In early stages of the work our attention was focused on the fate of the antibodies, as to their distribution and localization in the rat. For this purpose, the radioisotope I 131 was used as a tool for tracing the labeled antibodies and proved of great value for their detection *in vivo*, and for determining their affinity for the homologous organ. Although the localization of the antibodies in the various tissues could be interpreted by immunological principles of antigen-antibody reactions, the relative concentration of radioactivity in the respective organ offered direct evidence of the presence of antibody and its specificity.

In the course of our investigations phenomena were observed which pointed to the physiological responses of the rat injected with anti-organ sera as well as to functional changes of organs under the influence of the specific antibody. Some of the responses were demonstrated previously in the action of the anti-rat pancreas serum producing hyperglycemia in the injected rat (Anigstein *et al.*, 1954) and lately by the thyroid immune serum which lowered the basal metabolic rate in the injected rat (Anigstein, Eklund. Whitney, 1957). Of all the endocrine organs it was considered that the pituitary gland offers great opportunity for immunological approaches to the study of hormone antibodies.

In the immunological approaches to the study of the pituitary gland two types of antigens have been used by various authors, namely the total tissue homogenate (crude extract) or the purified hormones. It was shown by Rowlands and Young (1939) that the antiserum produced by the injection of crude extracts of anterior pituitary exhibits much greater antithyrotropic activity than the antiserum produced by the injection of a purified preparation. In the opinion of these authors the antihormones are anti-

bodies. The more recent investigations also point to the relation of antigenic potency with the degree of purification of the hormone concerned, for example Heijkenskjöld and Gemzell (1958) were unable to demonstrate antigenic properties of purified human growth hormone. To the contrary, Hayashida and Li (1958) have recently obtained physiologically active antiserum against purified pituitary growth hormone of bovine origin.

From the time of Collip's original anti-hormone theory (1935) which admits that every hormone may be antagonized by a specific anti-hormone, there has been a diversity of opinion as to the interpretation of the anti-hormones as antibodies. In the light of the recent developments most investigators are of the opinion that the anti-hormones belong to the complexity of antigen-antibody reactions (Werth 1956). The present study is limited to the anterior lobe of the rat pituitary which is used as antigen for the production of specific antibodies in the immunized rabbit. In spite of this limitation, the immunological responses of the rabbit to the structural complexity of the pituitary must be also highly complex and multiform. It is therefore possible that the main hormones of the pituitary such as the growth hormone, the thyrotropic hormone stimulating the thyroid, the adrenotropic hormone stimulating the adrenals, the gonadotropic hormones stimulating the gonads act as antigenic factors in the immunized rabbit and induce antibodies against the various endocrine substances. Should this be the case, then the injection of the pituitary antiserum (APS) into a rat could be followed by a suppression of growth, suppression of the thyroid functions, atrophy of the adrenal cortex, and reduction or atrophy of the gonads, thus simulating a "physiological hypophysectomy". These were the main criteria which were used during the present study for the evaluation of the effect of APS on the responses of the injected rat.

Material and Methods

a) *Immunization procedures*

Male adult rats of the Holtzman strain (from 300 to 400 gm.) were used for the source of the pituitary gland. After perfusion of the rat's head with Gey's balanced salt solution, the anterior lobe of the pituitary was separated, frozen in dry ice, and stored in deep freeze. The wet weight of the anterior lobe ranged from about 6 to 10 mg. as compared with the posterior lobe which weighed from 1.5 to 3.0 mg. For the immunization of rabbits, the

pituitaries were weighed and homogenized in a glass tissue grinder with a minimum amount of Gey's solution. For example: 66 mg. of tissue were homogenized in 1.5 ml. of Gey's solution. Freund's adjuvants were then added in a proportion of 1:1 and the preparation emulsified. Rabbits were inoculated with this preparation intradermally, 0.1 ml. into each foot-pad, containing about 10–12 mg. of the original tissue per rabbit. Each foot-pad was previously shaved and washed with aqueous merthiolate solution, 1:1000.

Prior to the inoculation of rabbits, 30 ml. of blood were removed by cardiac puncture for normal serum controls. Following the single intradermal injection, the rabbits were bled (40–50 ml. blood) in 3–4 weeks, the serum separated and stored in deep-freeze. After 15 to 20 days the rabbits were bled again. Intravenous booster shots of pituitary tissue were usually given after the second bleeding and after 10 more days the animals were sacrificed. A total of 23 rabbits were used in the immunization procedures.

The rats were inoculated intravenously (tail vein) or intra-abdominally either with APS or with normal rabbit serum either undiluted or diluted in single or multiple injections. In one series of rats, the lyophilized and reconstituted gamma globulin fraction of APS was injected. In most experiments, female rats weighing from 58 to 142 gm. at the beginning of the observation period were used. A total of 196 rats were used in the present work.

b) *Complement-fixation procedures*

The presence of antibodies in antisera was determined by complement-fixation tests, using the techniques developed by Witebsky (1955). The antiserum, inactivated for 30 minutes at 56° C., was serially diluted and pipetted in 0.1 ml. amounts into the standard complement-fixation tubes. The antigen consisted of homogenized tissue, usually 1:20 dilution freshly prepared the day of testing, centrifuged for 10 minutes at 1500 r.p.m., and kept in an ice-water bath during pipetting. Veronal buffer was used throughout the test for all dilutions. On some occasions three dilutions of antigen 1:20, 1:200, 1:2000 were tested with serial dilutions of antiserum. The normal serum obtained from rabbits prior to inoculation was tested with each corresponding series of immune serum. The antigen and two units of complement, each in 0.1 ml. amounts were added to the dilutions of antiserum, the tubes incubated 3 hours in the ice-box, followed by 1 hour at 37° C. in a water bath. The tubes were removed, placed in an iced bath during the addition of sensitized sheep cells, followed by further incubation until the controls were hemolysed. The racks were immersed in ice-water, the tubes subsequently centrifuged and the results recorded.

Experiments and Results

a. *Serological studies*

Complement-fixing antibodies for anterior pituitary antigen were found in the serum of twelve rabbits inoculated with the tissue. The titers ranged from 1:100 to 1:400 using serial dilu-

tions of the antiserum with 1:20 dilution of the antigen. Using the Witebsky technique of diluting the antigen, and 1:10 dilution of the antiserum, titers exceeding 1:2000 and as high as 1:10,000 were found. Example of the titration is seen in table 1.

TABLE 1

Complement Fixing Antibody Titers Using Anti-Rat-Pituitary Serum and Dilutions of Rat Pituitary Antigen

Rabbit Number	APS* Dilutions	Rat Pituitary Antigen Dilutions		
		1-100	1-1000	1-10,000
167	1:10	++++	++++	+
	1:20	++++	++++	0
	1:40	++++	++	0
	1:80	++++	++	0
	1:160	++++	++++	0
	1:320	++++	++++	0
163	1:10	++++	++++	+
	1:20	++++	++	0
	1:40	++++	++	0
	1:80	++++	++++	++
	1:160	++++	++	0
	1:320	++++	++++	0

* APS: anti-rat anterior pituitary rabbit immune serum.
++++ complete fixation.

To investigate the reactions of antiserum with heterologous anterior pituitary tissue, a limited study was made with rabbit and guinea pig pituitary tissue as antigens and the anti-rat-pituitary serum from immunized rabbits. The results as shown in table 2 indicate that there are complement-fixing antibodies developed in rabbit serum which react with components of anterior pituitary tissue of guinea pig and of rabbit. These findings indicate the rise of tissue specific components of the antiserum, as compared with the consistently negative reactions of the rabbit serum before immunization.

A series of tests were made to study the cross-reactions of the anti-rat-pituitary serum with other endocrine organs of the rat. Table 3 shows overlapping components of rat gonads and adrenals used as antigens with two anti-rat-pituitary sera. As shown in table, the titers for the specific tissues are approximately 10 times higher than those of the nonspecific tissue.

Investigations were made as to the possible changes in complement fixation titers due to multiple bleedings. Samples of

TABLE 2

Complement Fixing Antibodies in Anti-Rat Pituitary Serum for Pituitary Antigens From Other Species

APS* Dilution 1:10	Guinea Pig Pituitary Antigen			Rabbit Pituitary Antigen			Rat Pituitary Antigen		
	1:20	1:200	1:2000	1:20	1:200	1:2000	1:20	1:200	1:2000
165	++++	+++	0	++++	++++	0	++++	++++	++++
163	++++	++++	+	++++	+++	+	++++	++++	++++

* APS anti-rat anterior pituitary serum.
++++ complete fixation.

TABLE 3

Complement Fixation Reactions of Rat Antipituitary Serum with Rat Pituitary, Adrenal and Gonad

Rabbit Number	APS* Dilution	Rat Pituitary			Rat Gonad			Rat Adrenal		
		1:100	1:1000	1:10,000	1:20	1:200	1:2000	1:20	1:200	1:2000
	1:10	++++	++++	+++	++++	++++	++++	++++	++++	+
	1:20	++++	++++	0	++++	++++	+	++++	++++	0
171	1:40	++++	++++	0	++++	++++	0	+	0	0
	1:80	++++	++++	0	++++	++++	0	++++	0	0
	1:160	++++	++	0	++++	++++	0	+	0	0
	1:320	0	0	0	---	---	---	---	---	---
	1:10	++++	++++	+++	++++	++++	++++	++++	++++	0
	1:20	++++	++++	0	++++	++++	++++	++++	++++	0
169	1:40	++++	++++	0	++++	++++	0	++++	++	0
	1:80	++++	++++	0	++++	++++	0	++++	0	0
	1:160	++++	0	0	++++	++++	0	+	0	0
	1:320	+	0	0	---	---	---	---	---	---

TABLE 4

Complement Fixation Titers Following Multiple Bleedings

APS Dilutions	Rabbit Number and Date of Bleeding									
	108				162					
	Jan. 1*	Feb. 5	Feb. 19	Mar. 11	Jan. 1*	Feb. 5	Feb. 13	Feb. 22	Mar. 11
1:4	0	++++	++++	++++	0	++++	++++	++++	++++
1:8	0	++++	++++	++++	0	++++	++++	++++	++++
1:16	0	+++	+++	+++	0	+++	+++	+++	+++
1:32	0	+++	+++	+++	0	+++	+++	+++	+++
1:64	0	+++	+++	+++	0	+++	+	0	++
1:128	0	+++	+	+	0	++	0	+	0
1:256	0	+++	0	0	0	0	0	0	0

* Serum prior to immunization.
+APS

serum from each of two rabbits were obtained at different dates and kept frozen at −10°C. The results shown in table 4 indicate a drop in titer of circulating antibody after the initial bleeding.

b) *Growth rates of immature rats*

The effect of APS on the growth rates of immature female rats was conducted on a total of 196 rats divided in seven series of experiments under various conditions of serum administration.

Experiment 1. To investigate the effects of repeated intra-abdominal injections of APS on the growth rate as compared with the controls. A total of 25 rats 28 days old were divided into four groups: A). Six rats, average weight 78.5 gm., were untreated and served as controls. B). Six rats, average weight 80.1 gm., were injected with normal rabbit serum (0.5 ml. serum in 0.5 ml. sterile Gey's balanced salt solution) injected intra-abdominally 3 times weekly. C). Seven rats, average weight 74.5 inoculated with APS No. 180 (0.5 ml serum in 0.5 ml. sterile Gey's solution) intra-abdominally 3 times weekly for 2 weeks. D). Six rats, average weight 73 gm. were inoculated with APS No. 162 (0.5 ml. serum in 0.5 ml. sterile Gey's solution) intra-abdominally, three times weekly for 2 weeks.

Results. The growth rate curves showed a slight separation of the APS series from the controls beginning with the 6th day of observation. This separation increased during a 14 day period after which, a convergence of all 4 curves was noted. This convergence may indicate that the initial suppression effect of the APS on the growth rate may have been affected by the developing antibodies in the rat against the injected rabbit sera, as a result of repeated small doses.

Experiment 2. To investigate whether the formation of antibodies in the rat against normal rabbit serum would influence the growth rate of rats subsequently injected with APS. This experiment involved four groups of rats, each group consisting of six. A). These rats served as control and were left untreated (average weight 78 gm. per rat). B). Rats of the same weight were pretreated with pooled normal serum (0.2 ml. subcutaneously, 3 times for one week, followed by 0.5 ml. of APS intra-abdominally twice daily for 5 days. C). Rats pre-treated with normal rabbit serum as in group B, but followed by another injection of normal rabbit serum (0.5 ml. intra-abdominally, twice daily for 5 days). D). Rats were injected directly with APS without pretreatment, twice daily for 5 days.

Results. The growth rate curves of the four series showed an upward identical course during the first eight days when normal rabbit serum was injected on the first, the third and fifth day of

observation. This trend continued for the untreated group (A) only, whereas a leveling off deviation in the growth curves for the other three groups (B, C, D) took place on the eighth day when APS was administered to group D. The suppression of growth was relatively more accentuated in the group treated with APS, however following the first injection only, while no influence on the growth rate of the second injection on the 10th day and the third injection of APS on the 12th day was noted. This observation seems to indicate that the pretreatment with normal rabbit serum against which the rats became sensitized weakened the action of APS.

Experiment 3. To study the effect of the intravenous injection of the globulin fraction of APS. For this purpose 25 rats 29 days of age were divided into four groups: A). Six rats, average weight 74.6 gm. each, were untreated controls. B). Five rats, average weight 76 gm. each were injected into the lateral tail vein with APS (No. 171), 0.5 ml daily for 5 days. C). Seven rats, average weight 76.5 gm., were injected with the globulin fraction of normal rabbit serum as in group B. D). Seven rats, average weight 75.3 gm. were injected with the globulin fraction of APS (100 mg. per ml. distilled water) intravenously, 0.5 ml. daily for four days.

Results. The body weight curves of the two groups of rats (C and D) injected with normal or with antipituitary globulin fraction showed an almost parallel course which did not significantly differ from the untreated controls (A). All three curves finally converged at the end of the three weeks observation. On the other hand, group B treated with whole antipituitary serum (No. 171) responded to the injection by a suppression of weight, beginning with the third day until two out of the five rats belonging to group B died following the last intravenous injection. The other three rats survived until the end of the experiment showing 15 percent lower weight as compared with the untreated controls.

Experiment 4. To study the effects of daily intravenous injections of APS on the growth rates of immature rats. In this experiment 24 female rats, 29 days of age, were divided into four groups. A). Six rats, average weight 85 gm., were injected daily with 0.5 ml. saline. B). Six rats, average weight 88 gm., were injected with 0.5 ml. normal rabbit serum daily for five days. C). Six rats, average weight 87.1 gm., were injected once daily for five days with 0.5 ml. APS No. 169. D.) Five rates, average weight 91.6 gm., were injected with 0.5 ml. APS twice daily for five days. All injections were made intravenously into the lateral vein of the tail with 1 ml. syringes fitted with No. 26 needle. The rats were lightly anestheti'ed with ether during the process.

Results. No conclusions can be drawn from the course of the weight curves since the response to the daily injections of the sera did not differ to a measurable extent from the controls injected with normal rabbit serum or with saline. It is probable that the daily injections of rabbit serum resulted in sensitization of rats to rabbit serum proteins and provoked in group D injected 2X daily an increase in body weight.

Experiment 5. Purpose: To study the effect of a single massive dose of APS reconstituted from lyophilized state on the growth of young rats. The experiment involved 25 female rats averaging about 143 gm. in weight. They were divided into two groups of which 13 rats (group A) about 50 days of age (average weight 143.5 gm.) were each injected with a single dose of 2.5 ml. lyophilized APS (pooled from rabbits 171 and 172) reconstituted (83 mg. to 1.0 ml. distilled water) to normal constituency. The complement fixation titers for both sera were 1:160. The other group of rats (group B) averaged 142.6 gm. in weight. Each was injected with a single dose of 2.5 ml. pooled normal rabbit serum reconstituted from lyophilized state to normal constituency with distilled water (74.1 mg. lyophilized serum to 1.0 ml. water).

GROWTH-RATE CURVES OF RATS (GROUP A) INOCULATED WITH 2.5 ml OF RAT ANTI-PITUITARY SERUM (APS) AS COMPARED WITH GROWTH-RATE CURVES OF GROUP B INOCULATED WITH NORMAL RABBIT SERUM.

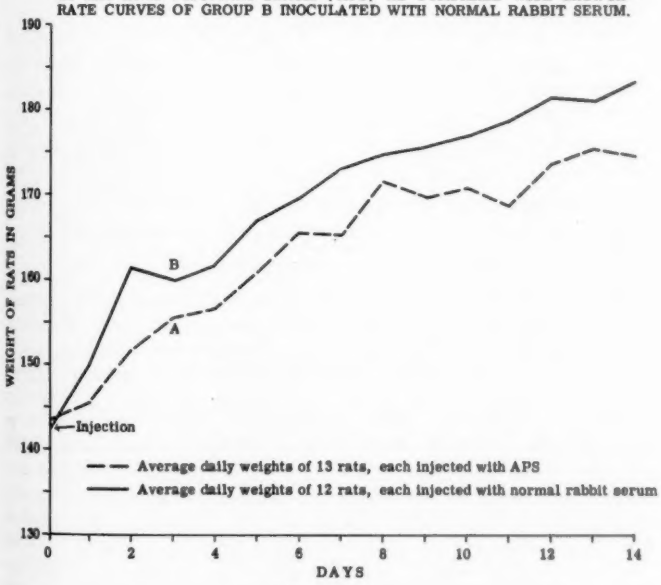

FIGURE I

Results. (Fig. 1) The study of the growth rate curves of each group of rats shows a separation of the curves from the beginning of the experiment. This separation continues during the entire period of observation with a general upward trend, almost parallel to the growth rate curve B, but at a lower level. The average final weight gain of group B injected with normal rabbit serum amounted to 40.4 gm. as a difference between 142.6 gm. at the beginning and 183.0 gm. at the end of the observation period. At the same time the differences in average total weight gain in group A injected with APS was 31.2 gm. (143.5 gm. compared with the final average weight 174.7 on the 14th day).

Experiment 6. To study the effect of a single intra-abdominal injection of APS (raw serum) on the growth rates of immature rats. For this purpose 15 female rats about 30 days old of matched weights, averaging 79 gm. each were divided into 3 groups of 5 each. The observation period covered a period of 27 days. A). The rats of this group were given a single intra-abdominal injection of 1.25 ml. of APS No. 177 (complement fixation titer of 1–80). B). Each rat was given a single intra-abdominal injection of pooled normal rabbit serum. C). The rats of this group served as controls and were given a single intra-abdominal injection of 1.25 ml. sterile Gey's solution.

Results. During the observation period of 23 days the APS series of rats followed the general trend of the growth rates for other groups, except for a suppression of weight on the seventh day and the fifteenth day after inoculation. These reactions were, however, temporary, and all three curves showed no significant differences. It was concluded that the dose of the serum (1.25 ml.) and a relatively low complement fixation titer (1:80) were inadequate, and it was decided to increase the dosage and to use another APS of higher complement fixation titer.

Experiment 7. In this series the effect of repeated injections of large doses of APS at weekly intervals was studied. For this purpose 48 female rats, about 28 days of age and 58–66 gm. in weight were divided in four groups of 12 each. A). The 12 rats were injected intra-abdominally with 2.0 ml. sterile Gey's solution on zero day of the experiment. A second injection of 2.0 ml. was given on the 5th day and a third injection of 2.5 ml. was administered on the 12th day. B). The twelve rats were injected with 2.0 ml. intra-abdominally with normal rabbit serum (No. 185) each on zero day of the experiment, followed by a second injection of 2.0 ml. on the 5th day (No. 182) and a third inoculation of 2.5 ml. (No. 6) on the 12th day. C). These 12 control rats were left untreated. D). In the attempt to establish a LD_{50} of the toxic effect of APS it was found that the dose of 2.5 ml. injected into young rats intra-abdominally caused death within 24 hours, while the dose of 2.0 ml.

was well tolerated. Therefore, the initial dose of 2.0 ml. was adopted for 25 day old rats. All 12 rats were injected with 2.0 ml. of APS each, followed by second injection on the 5th day. Under this mode of administration, the third dose of 2.5 ml. given on the 12th day was well tolerated most likely due to previous sensitization. The complement fixing antibody titer was 1:300 for the APS No. 183 and 1:160 for APS No. 186. All four groups of rats showed a steady gain in body weight. This trend continued until the fifth day

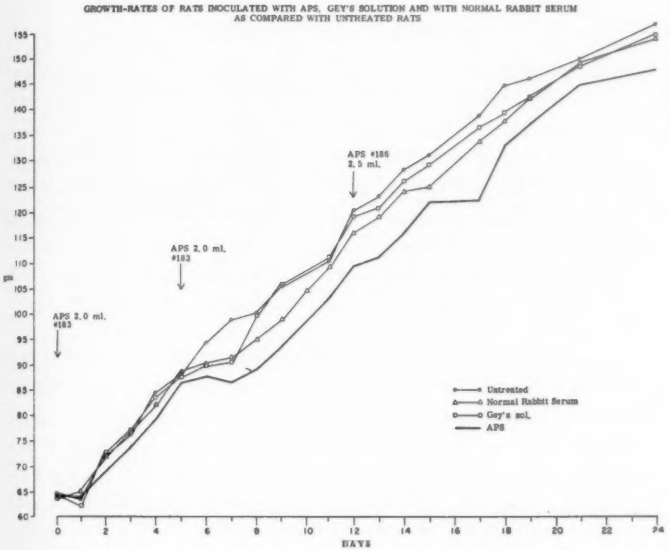
GROWTH-RATES OF RATS INOCULATED WITH APS, GEY'S SOLUTION AND WITH NORMAL RABBIT SERUM AS COMPARED WITH UNTREATED RATS
APS 2.0 ml. #183
APS 2.0 ml. #183
APS #186 2.5 ml.
Untreated
Normal Rabbit Serum
Gey's sol.
APS
DAYS

FIGURE II

when a sharp deviation of the growth rate curve of the APS group injected with APS took place. A definite separation of the curve representing APS from the other three curves was noted thus showing a relative inhibition of growth which was not affected by normal rabbit serum.

Results. Beginning with the sixth day following the last injection of APS, a distention of the abdominal wall in the 12 rats was noted. The autopsy of these rats showed in nine out of 12 a profuse abdominal hemorrhage ranging from 10 to 60 ml. of fresh unclotted blood. This blood contained no fibrinogen nor platelets and was found sterile, by culture and animal injection. The histological section of the fibrotic spleen showed normoblasts and

megakaryocytes, indicating extramedullary blood formation and severe vascular lesions. In addition, extensive adhesions of the liver, spleen and omentum were observed in the rats injected with APS. This group of rats showed signs of anemia with hemoglobin as low as 4 gm. and 2,100,000 red cells. The study of the relation of this phenomenon to the role of the hypaphysis in hemoporiesis and the influence of the pituitary antibodies will be continued.

Summary and Discussion

In the present study anterior rat pituitary was used as antigen for immunization of rabbits and production of anti-pituitary immune serum. Freund's adjuvant was added to the tissue homogenate and the emulsion injected into rabbit footpads. The single injection elicited specific antibodies after 3–4 weeks. Complement fixation tests indicated titers up to 1:2000 with the homologous antigen with overlapping and weaker fixation with rat gonads and adrenals. Complement fixation of rat pituitary antisera with pituitary tissue of rabbit and guinea pig suggests organ specificity.

Seven experimental series totaling 196 female immature rats were injected with various dosage of anti-rat-pituitary serum (APS). Evaluation of antiserum action was based on the study of the growth rate of the injected rats. In general, there was a moderate suppression of weight of rats treated with a single injection (2.5 ml.) of APS during the 20 days observation period. Similar lowering of body weight was observed in rats injected with APS three times at weekly intervals. The relation of the reduced growth rate with a possible disfunction of the pituitary demands further investigation. Anemia and profuse abdominal hemorrhage was observed in a series of rats injected intra-abdominally with APS. The unclotted blood showed no fibrinogen or platelets. Sections of the spleen showed normoblasts and megakaryocytes, indicating extramedullary blood formation and severe vascular lesions. The study of this phenomenon and its relation to the influence of the pituitary antiserum on hemopoiesis will be continued.

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[a] From the Departments of Preventive Medicine and Anatomy, The University of Texas Medical Branch, Galveston, Texas. This work was supported by a contract (NR 130–981) between the Office of Naval Research, Department of the Navy, and The University of Texas. Paper accepted for publication May, 1958.

THE VARIABILITY OF THE SPECTROPHOTOMETRIC TECHNIC FOR THE CLINICAL ESTIMATION OF SERUM ENZYME ACTIVITIES[a]

PAUL L. GAERTNER, E. L. MOSSER, AND LAWRENCE J. MILCH

Steinberg (5) reported a standard deviation of ± 6 per cent in the determination of serum glutamic oxalacetic transaminase (SGOT) on 10 replicates over the range of 44–53 units/ml., and, apparently, from his plotted data, an SGOT temperature coefficient equal to roughly 7½ per cent per degree centigrade. Chinsky *et al.* (2) noted that duplicate samples were consistently reproducible within 10 units/ml. of serum; however, no information was furnished on the range of activities over which such precision was attained. Rudolph and his co-workers (4) made special note of a significant temperature coefficient of enzyme activity above 25° C. but failed to quantitate it. Such fragmentary information seems to be about all that is available relating to the variability of clinical enzyme analysis by the spectrophotometric technic. It was decided, therefore, to introduce a special study into the laboratory routine at this facility and determine the precision to which serum enzyme results are ordinarily duplicated, taking precautions to minimize instrument and personnel bias.

Methods

The serum activities of glutamic oxalacetic transaminase (SGOT) and glutamic pyruvic transaminase (SGPT) were measured according to the method of Karmen *et al.* (3). Malic dehydrogenase (SMDH) and lactic dehydrogenase (SLDH) were determined by a procedure devised by Albaum (1). Beckman DU spectrophotometers No. SN 89039 (with photomultiplier) and SN 602 (without photomultiplier) were operated alternately by two of us (PLG and ELM)[b] on a statistically balanced schedule throughout the study. Rabbit serum was utilized as the enzyme source, and the evaluation was accomplished by replicate analysis at 4 levels of activity for each of 4 different enzymes, sacrificing a new rabbit[c] each day and studying the next higher level on successive days of a single week. For example, on day No. 1 it was proposed to evaluate SGOT within the activity range corresponding to less than 10 units of OD change per 5-minute reading period. Sixty cc. of blood were taken from the rabbit by cardiac puncture and the serum obtained after low-speed centrifugation. A preliminary enzyme assay was run im-

mediately, in duplicate, in order to determine activity, and then the pooled serum was diluted with 0.1 M phosphate buffer, pH 7.4, in order to bring the activity to the desired level. In many instances, dilution was unnecessary. The pooled material was then divided into 12 aliquots and stored at 10° C. Aliquots were removed from the refrigerator successively and each assayed, in duplicate, for enzyme activity between the hours of 9:00 a.m. and 5:00 p.m. by one or the other of the two people, using the two spectrophotometers on a scheduled basis. Serum blank values were recorded for each aliquot by taking simultaneous readings on a cuvette containing all reactants except the appropriate substrate (α-ketoglutarate, in the instance of SGOT). Temperatures were recorded to the nearest 0.1° C. in the cuvette chamber immediately prior to each assay. In day No. 2, SGOT was similarly evaluated for that activity corresponding to the range of 11–20 units of OD change per 5-minute reading period. This process was repeated until data had been accumulated on 4 enzymes over the ranges of activities ordinarily encountered in clinical research.

Discussion

The data recorded in table 1 indicate the per cent precision, that can be expected in routine enzyme analysis by the spectrophotometric method. With reference to the table and a specific

TABLE 1

Per Cent Precision (95 Per Cent of All Duplicates) at 4 Levels of Activity in the Determination of 4 Enzymes

Activity		Per Cent Precision			
Units of OD Change/Reading Period Range	Average Value	SMDH	SLDH	SGPT	SGOT
0–10	7	11	13	16	22
10–20	15	8	7	13	17
20–30	25	6	7	10	7
30–40	35	9	3	12	8

instance, these data may be interpreted as follows: it is noted that in the activity range of 0–10 units of OD change per reading period, SGOT manifests precision of 22 per cent. Thus, if each of 100 serum samples in this range was assayed for SGOT activity, in duplicate, 95 of the duplicate sets would agree within 22 per cent. These estimates are probably conservative since they are based on data taken in a single laboratory. Somewhat wider variability could be expected if a similar study was accomplished on an inter-laboratory basis.

It would seem that SGOT and SLDH measurements are better reproduced, on a per cent basis, at higher activity levels. On the

other hand, increased enzyme activity is associated with a significantly greater absolute error in SGPT and SMDH analyses. The reason for this variable result is obscure. That the change in the slide-wire calibration on the DU spectrophotometer at an OD of 0.4 was not involved was established by recording the range of all readings and demonstrating statistically that readings to the nearest 0.001 OD unit could be reproduced with equal precision from 0.504 to 0.147.

The data recorded in table 2 demonstrate that little, if any, bias

TABLE 2

Means and Standard Errors That Demonstrate Lack of Personnel and Instrumental Bias

	Number of Determinations	Mean	Standard Error of Each Mean	Significance (Level of Difference)
Technician A	96	20.91	0.06	...
Technician B	96	21.03	0.06	0.08*
Machine 1 (SN 89039)	96	20.96	0.06	...
Machine 2 (SN 602)	96	20.98	0.06	0.41*

* Not significant.

can be traced to either technician or spectrophotometer. Furthermore, the narrow slit width that can be achieved by use of a photomultiplier attachment to the DU spectrophotometer fails to reduce variability. On the other hand, temperature measurements over a narrow range (28°–31° C.) confirmed, for 3 of the enzymes, the profound effects noted for SGOT by Steinberg (5) and Rudolph (4). An approximately equal coefficient of 5 per cent per degree centigrade was recorded for SGOT, SGPT, and SMDH; however, the present data failed to record a measurable temperature coefficient of activity for SLDH.

The practical impact of these data is best viewed in terms of the criteria established by Chinsky (2) for normal (up to 40 units/ml. of serum), borderline (40–50 units/ml. of serum), and high (above 50 units/ml. of serum) SGOT measurements in humans. If a sample with an actual SGOT activity of 45 units/ml. is analyzed in St. Louis at 77° F. and also in San Antonio at 84° F., the temperature difference could combine with the inherent variability of the method to yield results of 37 and 53 units/ml., respectively, that fail to differ significantly ($P>0.05$). Such, of

course, still does not include the instrumental and personnel variability that is inevitable between laboratories.

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NOTES

[a] Department of Pharmacology and Biochemistry, School of Aviation Medicine, USAF, Randolph Air Force Base, Texas. Paper accepted for publication March 23, 1958.

[b] Extensive experience had been gained with the spectrophotometric technic during the previous 12 months, during which time several hundred assays had been done by each man.

[c] The higher activities were induced in the rabbit serum by prior exposure of the animals to low concentrations (circa 100 ppm.) of CCl_4 vapor over a 4-hour period.

[d] Let a single sample be analyzed twice to yield values x and y—*either* successively by the same individual *or* by two different individuals.

$$\text{Per cent precision} = \frac{\dfrac{x - y}{x + y}}{2} \times 100$$

The authors are indebted to Dr. Harry Hughes, Department of Biometrics, School of Aviation Medicine, USAF, for the statistical evaluation of these data.

ANALYSES OF NORMAL AND ABNORMAL HUMAN-ANTIHUMAN PRECIPITIN REACTIONS IN AGAR COLUMNS

W. G. Glenn,[a] G. F. Lanchantin,[b] R. B. Mitchell,[a] and I. W. Marable[a]

The diffusion of antigens into an antiserum-agar medium permits analyses of precipitin systems and characterization of the components. Many studies demonstrate that the diffusion rate is primarily a function of antigen concentration and that the zone density is an indication of the amount of reacting antibody (Oudin, 1952, 1955; Becker, 1953; Munoz, 1954).

In general, it is a valid statement that most normal human sera will react similarly to the same antihuman serum in agar columns and should, therefore, show similar multizone diffusion patterns (Becker, 1953); conversely, abnormal sera should show aberrant diffusion patterns. A preliminary pioneer study indicated that these premises held promise for distinguishing between normal and abnormal human sera (Glenn, 1957). This report consolidates and extends the previous findings using other human sera and pooled rabbit antiserum. In addition to some prominent differences in the agar diffusion patterns, diffusion rates, and zone densities, a rapidly diffusing system that appears to be associated with mucoprotein (seromucoid) (Winzler, 1948; Weimer, *et al.*, 1950) in abnormal sera, was consistently observed.

Materials and Methods

External reactants.[c] These human sera were from 3 groups: 1) 47 individual samples from supposedly "normal" persons of both sexes who were applicants for routine physical examinations; 2) 8 individual samples from male patients hospitalized because of various conditions; and 3) 20 samples from 5 men receiving in-patient therapy for thermal and traumatic injuries.[d]

Antigen and antisera. A pool of normal human sera from at least 50 people was used to induce six rabbit antisera according to the method published elsewhere (Glenn, 1956).

From the six individual antisera, two were pooled. These showed the best zonation and differential density to the homologous antigen by agar column analyses and constituted the antihuman serum used throughout this report.

Serum agar columns. Detailed procedures for preparing the diffusion columns have been reported (Glenn, 1956). Several hundred tubes were filled from 4 separately prepared mixtures of internal reactant (antiserum plus agar). Separate mixtures are used so that the internal reactant does not remain in the sol state at 46° C. longer than 15 minutes. Each column was filled with approximately 0.20 ml. of internal reactant and stored at 4° C. for not more than 5 weeks. (The columns were selected later at random to scatter any differences in results due to possible dilution errors.) Rubinstein (1954) indicated that storage of this nature did not appreciably alter the diffusion rate; and a preliminary study (Glenn, 1957) showed that zonal density was less variable for prepared columns kept at 4° C. for 5 weeks than it was when the internal reactant was mixed anew for each analysis.

Columns with antigen were prepared in duplicate, stored at 24° C., and measured between 115–120 hours.

Chicken serum, used as a heterologous control, did not react with the anti-human serum. All antigens in the 3 groups were tested against normal rabbit serum-agar columns. The five normal and six abnormal human sera that reacted with this control were discarded.

Absorptions. The principal components of electrophoretically repurified ethanol fractions II and V (*vide infra*) were used to absorb repeatedly separate aliquots of the antiserum. Further details of the procedure are available (Glenn and Garner, 1957).

Mucoprotein (MP). This material was fractionated from pooled human serum by the method of Weimer, *et al.* (1950).

Paper electrophoresis. Zone electrophoresis on all external reactants was done as indicated by Glenn and Garner (1957) except that the bromphenol stained strips were exposed to ammonia vapors before scanning (Spinco Division, 1957).

Ethanol precipitated plasma fractions II and V were purified by continuous paper electrophoresis (.015 M sodium barbital—.003 M diethyl barbituric acid, pH 8.5, ionic strength .018) at 600 v, 10.5 ma (Glenn and Jaeger, 1957). The fractions were dialysed against saline for 48 hours at 4° C. then concentrated by evaporation.

MP was electrophoretically characterized by continuous paper electrophoresis (.002 M sodium acetate, .08 M sodium chloride, N/10 hydrochloric acid, pH 4.6, ionic strength 0.18) at 100 v, 12.75 ma. The flow path on the curtain was identified by the glycoprotein stain method of Köiw and Gronwall (Block, *et al.*, 1955). Purified fraction II was run simultaneously on the opposite curtain as a control.

C-reactive protein (CRP). All sera from hospitalized subjects were reacted with commercial C-reactive protein antiserum by a modification of the capillary tube technic of Anderson and McCarty (1950). Special care was taken to use only .015 ml. (3 cm. in 0.8 mm. tube) of each reactant. Materials were incubated at 37° C. for 2 hours, stored at 4° C. for 46 hours, then centrifuged at 850 r.p.m. for not less than 10 minutes until all aggregations were precipitated. The heights of the precipitates were measured at 20X

magnification with a previously calibrated optical micrometer mounted in a dissecting microscope.[e]

Quantitation of agar columns. Details of the direct photometric quantitation of columns by the Serum Agar Measuring Integrator (SAMI) are available (Glenn and Garner, 1957; Glenn, 1957).

In this study the vertical column speed was reduced to approximately one-half the chart rate by a variable ratio speed changer.[f] A linear calibrator, an electromagnetic accessory that operates from a cam on the SAMI, provided a column to chart rate calibration on the baseline of the SAMIgraphs. Measurements on the graphs could be converted to actual diffusion values by means of this calibration.

Experimental Results

Mucoprotein (MP). The fractionated MP showed a strongly positive orcinol reaction (Weimer, 1950; Winzler, 1955) and a horizontal anodic shift of 1.5 cm. in continuous paper electrophoresis at pH 4.6. Only one path was apparent. A cathodic shift of 0.5 cm. was shown by fraction II under the same conditions.

When diffused into the antihuman rabbit serum, MP showed three faint zones (optical density .015–.025) after 72 hours reaction time.[g]

Qualitative diffusion pattern analysis. When diffused into the antiserum, all the normal serum samples showed similar patterns when observed between 115–120 hours (Fig. I, patterns A, B). The zone nearest the antigen interface was the most dense; this was labeled number 1. Then followed a fainter zone designated 2. The next major zone, number 4, usually had prominent density characteristics and a diffuse leading edge (Becker, 1953) which resulted in a symmetrical graph for that zone. The pattern ended with zone 6 which had a smooth density gradient with an especially sharp leading edge.

As shown in figure I (patterns C-E) most abnormal sera also showed zones 1, 2, 4, 6; however, the optical densities (O.D.) of the number 4 and 6 zones were usually appreciably larger and there was a disproportionate shift in the entire diffusion pattern. Zone 4 in many abnormal sera was either crowded upon zone 6 or did not resolve. In the latter cases, zone 4 was apparently at the same locus as number 6. One normal and all abnormal samples except 2 (footnotes, table 1) also showed one faint zone in addition. This 6′ zone consistently appeared ahead of zone 6.

Zone identification. In a former report (Glenn and Garner, 1957) using another rabbit antihuman pool, an association of

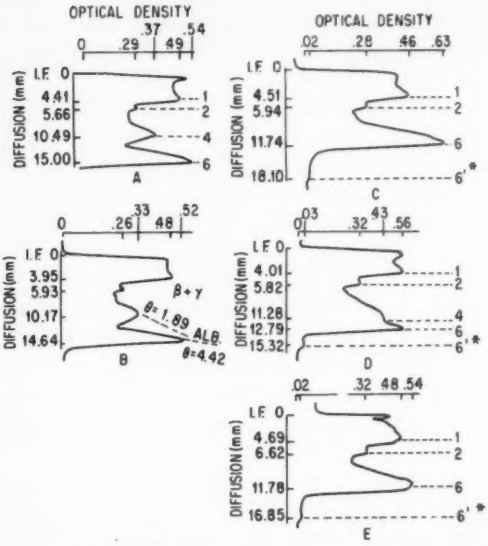

FIG. I. SAMIgraphic diffusion patterns showing prominent differences between normal human-antihuman and abnormal human-antihuman precipitin reactions in agar columns. Patterns A and B are typical normal serum patterns. In pattern B the turbidimetric density gradients for zones 4 (beta and gamma globulin) and 6 (albumin) are shown as tan Θ. Pattern C, the external reactant, was serum at 6.3 per cent protein, 18 days post-burn; pattern D, 5.6 per cent, 2 days post-burn; pattern E, 5.6 per cent, lung carcinoma. Note the crowding effect (pattern D) and the failure of the number 4 zone to resolve (patterns C, E). Abnormal patterns C-E also show the 6′ zone marked with an asterisk. Reaction time, 118 hours. (The 6′ zone has been qualitatively observed in some abnormal sera after 18 hours reaction time).

zones 4 and 6 with beta-globulin and albumin, respectively, was established by absorption. Using the same procedure and more purified plasma fractions, the results were the same (Fig. II). In addition, zone 4 was found to contain 2 systems—beta and gamma globulin. It was possible to absorb out the gamma globulin zone and uncover a narrow zone that would absorb out with minute amounts of beta-globulin. The control agar column with gamma globulin diffused into gamma absorbed antihuman serum was negative in these trials.

When the antiserum was absorbed with purified albumin until the control column was negative, there still remained a faint zone in the same position as the original albumin (zone 6). This faint

zone was designated number 6′ and disappeared following absorption with MP. In the abnormal sera in which zone 6′ was evident below the albumin, this zone was not seen when the antiserum was absorbed with MP.

Quantitation of diffusion patterns.[h] The turbidimetric density gradient or mean theta value, tan θ (Glenn, 1957) for zone 4 based on 26 normal sera was 1.92 ± 0.4 (range 1.13–2.92) and for zone 6, 4.03 ± 0.5 (range 2.88–5.01). The lower θ values for zones associated with globulin compared to albumin were in agreement with previous results describing this differentiating characteristic (Fig. I, pattern B).

Duplicate agar columns were directly compared with the respective SAMIgraphic records immediately after scanning. When the principal zones and their positions were marked, conversion of the figures to actual mean optical densities and mean

TABLES 1A and 2A

Comparison of the means (χ) and observed standard deviations (σ) of the average diffusion (D), D ratios and mean optical densities of the marker precipitin zones in the agar column analyses of normal and abnormal human sera between 115 and 120 hours at 22° C. C-reactive protein (C.R.P.) and total protein (T.P.) data are also included.

D of zones

Group	Data	1	2	4	6	6-4	6/4	6′	C.R.P. (mm)	T.P. (percent)
I Normal	n	48	48	48	48	48				38
	χ	4.14	6.34	10.30	14.27	3.96	1.39	0	0	7.15
	σ	.45	.49	.79	.52	.74	.09			.55
II Abnormal	n	5	5	4	5	5	4	4	5	5
(Post-burn)	χ	3.99	6.02	10.16	12.43	2.12	1.26	16.17	.96	5.9*
	σ	.28	.52	.91	1.20	1.60	.14	.67	.54	.99
III Abnormal	n	7	7	6	7	7	6	6	7	7
(Miscellaneous	χ	4.66*	6.27	11.84†	13.52	1.68†	1.17†	16.50	.66	6.07
conditions)	σ	.55	.80	1.25	1.07	.74	.10	.94	.41	.57

Optical densities of zones

Group	Data	1	2	4	6	6-4	6/4
I Normal	n	48	48	48	48	48	48
	χ	.49	.27	.32	.51	.19	1.62
	σ	.10	.03	.03	.03	.02	.15
II Abnormal	n	5	5	4	5	5	4
(Post-burn)	χ	.50	.29	.40	.54	.10*	1.32*
	σ	.04	.03	.08	.06	.07	.20
III Abnormal	n	7	7	6	7	7	6
(Miscellaneous	χ	.49	.29	.41†	.54†	.11	1.32†
conditions)	σ	.02	.02	.03	.02	.06	.11

* $P < .05$
† $P < .01$
D = Mean diffusion distance (mm) between 115 and 120 hours for duplicate columns.

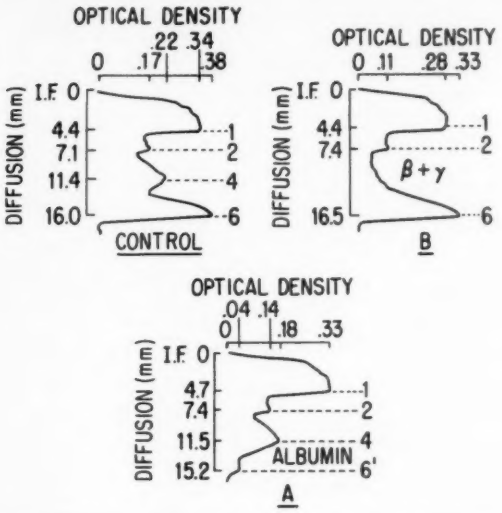

FIG. II. SAMIgraphic diffusion patterns of normal human-rabbit antihuman precipitin reactions in agar columns. The major marker zones are shown in the control pattern (upper left). In the control pattern the antiserum was "absorbed" with 0.20 ml. of 0.85 per cent saline. Pattern A, 1 ml. of antihuman serum completely absorbed with 0.125 ml. of normal serum albumin. Note the remaining 6′ zone. Pattern B, 1 ml. of antihuman serum completely absorbed with 0.20 ml. of fraction II (gamma globulin). A faint zone which can be absorbed with fraction III (beta globulin) is also at the number 4 locus. All antigens used for absorption contained 4.8 mg. N/ml. Reaction time, 113 hours.

diffusion distances provided the data for table 1. In general, the D6/D4 which represent the comparative diffusion of zones associated with albumin and globulins were noticeably less for abnormal sera compared to normal. This agreed with abnormal patterns where the number 4 zone was crowded into number 6 (Fig. 1, pattern D). When the 4 zone was not resolved at all and was obscured by number 6, it was, of course, not possible to get a D6/D4 ratio. Patterns of this type were considered definitely abnormal (Fig. I, patterns, C, E). In patient 1D (table 1), the sample was taken on the day of burn and apparently any serological imbalance, *i.e.*, altered D6/D4, 6′ zone was not as yet reflected in the serum. When the optical density of zone 6 is divided by number 4, the abnormals again showed a marked

GROUP	NO. PATIENTS	NO. SAMPLES	BURN INDEX%	D6/D4	O.D.6/O.D.4	D6'/D6	CRP (mm)
I NORMAL[1]	47	47	0	1.39± .09	1.62± .15	0	0
II ABNORMAL (POST BURN)	1A	12	50.0	1.14± .02[2]	1.10± .01	1.40± .06	0.88± .11
	1B	1	2.5	1.31	1.42	2.27	0.70
	1C	1	9.0	1.13	1.20	2.53	1.25
	1D	1	9.0	1.44	1.55	0[3]	0.14
	1E	5	21.0	1.18-1.20[4]	1.21-1.26	1.44± .17	1.29± .006
III ABNORMAL (MISCELLANEOUS CONDITIONS)	6F	6	0	1.17[5] ± .10	1.32± .11	1.24± .11	0.66± .41

TABLE 1

Quantiative differentiating characteristics of serum agar column diffusion patterns of normal and abnormal human sera reacted with pooled antihuman rabbit serum. C-reactive protein determinations are also included. Note the appreciably lower D6 D4 diffusivity ratios and the O.D.6 O.D.4 ratios for abnormal sera.

[1] Excludes 1 supposed normal that showed a 6′ zone and 0.2 mm CRP.

[2] In 4 samples the number 4 zone did not resolve and was apparently crowded into number 6.

[3] Sample was taken on day of burn; no 6′ observed.

[4] Based on 2 samples; in 3 samples the number 4 zone did not resolve.

[5] Does not include 1 patient whose sample had a normal pattern and no diagnosis was made after a thorough physical examination; excludes 1 patient with infectious hepatitis and old tuberculosis of the kidney but whose serum did not show a 6′ zone.

Miscellaneous conditions:
Carcinoma of lung, evere dermatitis, cirrhosis of liver, chronic renal and congestive cardiac failure, gallstones, and pancreatitis.

Burn index = estimated percent area of third degree burn plus one-fourth the estimated percent area of second degree burn.

difference from the normals. Exceptions to this are patient 1D again and 1B who had the lowest burn index.

Zone electrophoresis by the paper strip method was performed on all normal and abnormal samples. Table 2 shows these results which will provide some information relative to the degree of abnormality of the samples. Again samples 1B and 1D are more nearly within normal ranges for most serum components than other abnormal sera. These results generally agree with those found by agar column analyses. However, in considering individual samples, there was no apparent consistent relationship between the grams per cent of any of the serum components as

TABLE 2

Mean and standard deviations for total protein and serum components of normal and abnormal human sera separated by paper electrophoresis. The grams per cent represents only stainable protein with no correction for the albumin tailing effect.

Group	No. Patients	Samples	Total* Protein	Grams, Per Cent, Globulins			
				Albumin	Alpha-1	Alpha-2	Beta	Gamma
I Normal	43	43	7.19± .05	3.93±0.6	0.29±0.07	0.76±0.20	0.97±0.17	1.33±0.36
II Abnormal	1A	11	4.99±0.76	1.77±0.83	0.43±0.10	1.13±0.19	0.62±0.15	1.03±0.31
(Post burn)	1B	1	6.8	3.21	0.25	1.03	0.82	1.48
	1C	1	5.6	3.02	0.36	0.85	0.64	0.72
	1D	1	7.0	3.99	0.21	0.93	0.76	1.11
	1E	5	5.52±0.49	1.70±0.48	0.37±0.21	1.29±0.20	0.98±0.15	1.07±0.50
III Abnormal (Miscellaneous conditions)	6F	6	6.07±0.57	2.5 ±2.17	0.34±0.02	0.93±0.26	1.07±0.31	1.21±0.38

* Biuret method (20).

established by paper electrophoresis and the diffusion or optical densities of the precipitin zones in agar columns.

As an additional check, CRP determinations by a capillary tube modification (Anderson and McCarty, 1950) were also done on these abnormal sera. The results are shown in the right hand column (Table 1). Since the antihuman serum did not react with CRP, it is believed this abnormal protein did not form a zone in the human-antihuman patterns. Hence the 6′ antigen and CRP were different entities. Continuous paper electrophoretic analyses of the mucoprotein and CRP also showed that these were not the same substances. CRP had a mobility approximating alpha or beta at pH 8.6; MP had an isoelectric range below pH 4.6 since it migrated anodically under those conditions.

There was no apparent agreement between the degree of CRP reaction and the extent of abnormality as measured by the agar column method. Only in one instance (infectious hepatitis) was a CRP trace reaction noted and the agar column pattern similar to normal distribution. There is some evidence that in cases of hepatitis the MP level in the serum is decreased. This may explain why a 6′ was not evident in this sample.

The results of an analysis of a serum sample from one apparently healthy normal male are not shown in table 1. This sample showed a 1.26 D6/D4 ratio (below normal), 6′ zone (abnormal), and negative C-reactive protein. Three days later acute distress diagnosed as renal calculi required that the man be hospitalized.

Discussion

The relative position of zones associated with albumin and globulins are primarily the resultant of the diffusibility characteristics (concentration, symmetry, etc.) of the antigens compared with their respective antibodies (diffusivity ratio). Zonal densities decrease fairly linearly with dilutions of the antiserum (Oudin, 1952; Munoz, 1954) and are probably a good indicator of the amount of antibody in a zone. However, there is some evidence that the antigen concentration may also affect zonal density (Glenn and Garner, 1957). Since this is an inverse relationship, and many abnormal sera have differential hypoproteinemia, this factor may also be responsible for increased zonal densities in the pathologic sera.

The diffusion profile and subsequent quantitation of multizone human-antihuman reactions that enables differentiation of normal from abnormal sera by agar column analyses of the whole serum is a new approach in serum agar column analyses. The differences observed are probably due, in part, to the phenomena of "allotypy" of certain protein antigens indicated by Oudin (1956). In this concept a protein family, *e.g.*, albumin with its narrow isoelectric range, salt sensitivity, etc., could be composed of minutely different molecular configurations in different individuals. Such variations might account for differences in specificity, diffusibility, and interaction with the internal reactant and thus result in a different SAMIgraph for the abnormal sera.

Possible differences between the normal and abnormal sera other than zone forming antigens such as the influence of inorganic ions, concentration of non-specific proteins, slight pH differences and other factors on the pattern are yet to be demonstrated. The results presently reported indicate that the normal and abnormal patterns differ and such differences do not appear to be correlated with C-reactive protein or paper electrophoresis.

Elevated MP or seromucoid levels in thermal injuries, trauma, cancer, and other conditions have been shown by biochemical studies (Winzler, 1955; Lanchantin *et al.*, 1956). The appearance of the 6′ zone below the albumin (zone 6) is a function of the serological balance between the external and internal reactants. It is possible to see a 6′ zone below albumin in normal sera when the antiserum is not standardized by selection. For this reason, the antihuman rabbit pools with which this phenomenon has been observed have each been "standardized" by first establishing a basic pattern with 47 individual normal human sera. This procedure would always be necesary when one is measuring an abnormal concentration of a normal serum component.

Conclusions. Human sera differ: in their affinity for homologous rabbit antibody, diffusibility against antibodies, and the photometric pattern made in serum agar columns at 115–120 hours reaction time.

The SAMIgraphic profiles of normal human-rabbit antihuman sera are essentially similar. Some abnormal sera reacting

with the above antiserum have agar column diffusion patterns that are for the most part, markedly different from normal sera. Such differences are: in the comparative diffusion of zones apparently associated with certain globulin and albumin fractions, the optical density ratios of these zones, and—with standardized antisera—the appearance of a fast diffusing precipitin system apparently associated with mucoprotein (seromucoid).[4]

Appendix A

The data presented below are statistical analyses for numerical values derived from SAMIgraphic patterns. To avoid weighting the data in those abnormal patients in which more than one sample was analysed, only the specimen most nearly representing the mean for that patient was considered. Thus, although 20 samples from burn patients were observed, these came from "n" or 5 individuals.

In addition to the quantitative differences mentioned in the text, the relatively high negative correlations of the $\overline{D}$ of zone 6′/6 with total protein for the abnormal groups (Tables 3C, 3D) suggests that the concentration of antigen(s) forming the 6′ zone was independent of the total protein. Stereoisomeric and stoichiometric inequalities in molecular configurations and combining capacities resulting in varying antigens specificity (allotype) may account for the opposite correlations of $\overline{D}4$ and $\overline{D}6$ with total protein (Tables 3A, 3C, 3D) shown for normal and abnormal samples.

TABLES 3A, 3B, 3C, 3D

Correlation coefficients of diffusion distances (D) with total protein (T.P.), and optical densities (O.D.) of zones for normal and abnormal human sera by agar column analyses.

3A

I. Normal Group

D of zone

	2	4	6	6/4
D zone 4	.45‡		.42‡	
D zone 6	.54§			
T.P.		—.16	—.32*	.02

3B

I. Normal Group
O.D. of zone

	2	4	6
O.D. of zone 4	.40‡		.69§
O.D. of zone 6	.66§		

3C

II. Abnormal (Post burn)
D of zone

	4	6	6′/6
T.P.	—.27	.91†	—.83

3D

III. Abnormal (Miscellaneous conditions)

D of zone

	4	6	6′/6
T.P.	.43	.82†	—.67

* P ~ .05
† P<.05
‡ P<.01
§ P<.001

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[c] Substances layered on top of the antiserum-agar in columns.

[d] Kindly supplied through the courtesy of the U. S. Army Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Texas.

[e] Differences in the character of the centrifuged precipitates between the standard CRP antigen (positest) and the human sera when examined at 20X precluded extrapolating actual CRP concentrations in the human sera.

[f] Metron Corporation, Denver 9, Colorado.

[g] In ultracentrifugation, this MP gave a single peak at intervals up to 80 minutes at 259,700 x G. Courtesy of the Department of Biochemistry and Pharmacology, School of Aviation Medicine, USAF, Randolph AFB, Texas.

[h] For statistical correlations of zonal diffusion with total serum proteins and comparison of mean diffusion and optical densities, see Appendix A.

[i] Since the publication of this work, 50 "normal" and 140 "abnormal" human sera have been analyzed by the same method with similar results. Complete details will be published later. Accepted for publication April, 1958.

ACTION OF CIGARETTE TAR AND SMOKE ON CHEMICALLY INDUCED CARCINOGENESIS

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Chemicals in cigarette smoke may act as non-carcinogenic synergistic agents to intrinsic lung cancer in human populations (13, 30). Synergistic effects of smoke toward tumor induction might parallel similar instances found in laboratory animals (2, 27). However, examples of carcinogenic antagonism, or inhibition of tumor induction by carcinogenic and non-carcinogenic agents, have been observed (11, 12, 14, 15, 16, 22, 23, 28). The existence of the phenomena of synergism and antagonism suggests a new method for the study of the effects of tobacco tars and smoke on cancer induction.

It was of interest to determine if exposure to cigarette smoke or smoke tar preparation would either enhance or inhibit the course of a well-established carcinogenic sequence, namely that resulting from the feeding to rats of diets containing the liver carcinogens 2-diacetylaminofluorene (DAF) or 3′-methyl-4-dimethylaminoazobenzene (3′MeDAB). Within certain conditions, a definite delaying action of cigarette smoke and tar was found on the development of hepatic malignancy induced by 2-diacetylaminofluorene. In contrast, the precancerous state induced by 3′MeDAB was not inhibited by the use of tobacco tars or smoke.

Methods

Two groups of Sprague-Dawley strain male rats were fed semi-synthetic diets containing 2-diacetylaminofluorene (0.05 per cent) for periods of two and three months. One group was exposed to cigarette smoke. Smoke was obtained from standard filterless cigarettes by the use of an automatic smoking machine. The machine was designed to permit independent puffs of smoke to be expelled into a wooden chamber which housed the animals. The machine consisted of a bellows actuated by a variable-speed electric motor, the cigarettes being automatically changed at given intervals. In the first series of experiments, the dose was twenty cigarettes an hour for eight hours each day for 12 to 14 weeks. In subsequent experiments on smoke, the dose level was reduced to five cigarettes per hour for eight hours each day.

A second group of rats was injected with a crude tar preparation intraperitoneally on alternate days. The tar preparation was obtained by the burning of cigarette tobacco in a glass funnel, and drawing smoke by suction into a flask immersed in a solid carbon-dioxide and acetone bath. The resulting condensed tar was washed in water, dissolved in hot acetone, and filtered. The filtrate was poured into a large volume of cold water and thoroughly washed in 0.1 N HCl. This was followed by several washings with water and drying on filter paper. It was stored until needed at room temperature in a sealed container. For administration to rats, the material was suspended in peanut oil at a concentration of 50 mg./ml. The dose administered was 0.1 ml. every other day, delivered intraperitoneally. Controls received peanut oil only.

The authors realized that the method of preparation of the tar may not have produced tars identical to those obtained by human smoking. However, before the above studies were completed, a tar fraction became available which was isolated under physiologically simulated conditions (Eusta Paper Corporation, Pisgah Forest, North Carolina). This tar fraction was tested in a similar fashion to that used for the crude tar of the earlier studies.

For these tests, male albino rats were fed synthetic diets containing either 0.06 per cent 3′MeDAB or 0.04 per cent DAF. In these series, the animals were maintained on these diets until a fairly high percentage of tumors was evident in the controls. This is in contrast to the previously mentioned groups, where the rats were maintained for 8 to 14 weeks only.

Other rats which were fed diets containing the carcinogens were painted with the tar condensate twice weekly. The tar was painted on shaved areas on the back of the neck.

The levels of hepatic riboflavin were determined by a fluorometric analysis of the pyridine-acetic acid eluate from Florisil columns as previously described (1, 3, 8). Analysis of deoxyribose nucleic acid employed the hot trichloroacetic acid method of Schneider (26) and the colorimetric determination of Dische (5).

The preparation of the carcinogen, 2-diacetylaminofluorene (DAF), followed the procedure of Morris *et al.* (21), but used instead of aminofluorene, 2-acetylaminofluorine as the starting material. The final material was recrystallized twice from hot 95 per cent ethanol and had a final melting point of 133–135° C. (uncorrected). The assay method of estimating bound dye is that of Miller *et al.* (17).

Powdered norit was washed successively in hot 0.1 N HCl, 0.1 N NaOH, distilled water, hot 95 per cent ethanol, and ether. The dried product was suspended in peanut oil at a concentration of 50 mg./ml. and injected intraperitoneally at a dosage of 0.1 ml. per rat every other day.

Gross observations were made at the time of sacrifice. Increasing precancerous damage was indicated by an increased number of pluses. Large tumors were rarely seen at the early periods studied. The amount of feed consumed was measured. No differences in intake were observed between the treated and control rats.

Results

In a study of the inhibitory nature of a substance against tumor development, it is important to attempt a quantitative measure of the inhibition. To this end, a preliminary series of analyses were performed on livers of rats fed DAF for varying time periods. The levels of liver riboflavin and liver deoxyribose nucleic acid were determined, since Griffin *et al.* (9) had previously demonstrated a concurrent reduction in hepatic riboflavin concentration with no change in deoxyribose nucleic acid (DNA) during the feeding of acetylaminofluorene. It was desirable to establish whether the same phenomenon resulted with DAF and if the concentrations could be utilized as a measure of liver damage.

The results are described in Table 1. It may be noted that the concentration of DNA did not increase during the feeding of diets

TABLE 1

The Effect of 2-Diacetylaminofluorene on Liver Deoxyribose Nucleic Acid and Riboflavin

Time on Diet	No. of Rats	DNA mg. Per Cent	Riboflavin Microgm. per gm.	Gross Damage	Histological Comments
8-12 weeks basal	9	310±40	24.9±1.0	(—)*	Normal appearance
2 weeks DAF	3	no data	25.9±2.6	(—)	Normal appearance
6-8 weeks DAF	4	332±20	16.8±2.1	(++)	Fatty metamorphosis Minimal cirrhosis
11-12 weeks DAF	5	308±17	15.8±0.9	(+++)	Cirrhosis
18 weeks DAF	2	280±25	14.3±1.1	(+++)	.....
23 weeks DAF	2	311±21	13.5±0.0	(+++)	Bile duct proliferation, stomal thickening, bile duct cysts, hepatoma foci

* For interpretation of designations, see METHODS.

containing DAF. Consequently DNA was not utilized as a measure of the extent of change toward malignancy. However, the riboflavin concentration did decrease markedly sometime after two weeks of feeding, and by the sixth week the level had decreased to approximately two-thirds of the original value. Histological examination generally demonstrated cirrhosis at the sixth to eighth week period, and gross examination permitted suitable evaluation of the precancerous state. Hence, the majority of sub-

sequent studies utilized the eight-week feeding interval as the test period.

Three groups of animals were exposed to cigarette smoke (Table 2). The first series consisted of rats fed the carcinogenic

TABLE 2

Effect of Smoke on Liver Riboflavin and the Development of the Precancerous State in Rats Fed 2-Diacetylaminofluorene

Experiment	No. Survived/ No. Started	Wt. at Sacrifice/ Wt. at Start	Riboflavin Microgm/gm.	No. of Rats Showing Gross Damage*
Group 1, 14 Wks				
DAF only	4/10	185/159	14.8±0.5	3 (+) 1 (+++)
DAF + Smoke	10/10	165/126	16.5±1.9	7 (—) 3 (+)
Group 2, 8 Wks				
DAF only	10/12	186/153	18.3±1.7	3 (+) 4 (++) 3 (+++)
DAF + Smoke	15/15	192/159	20.3±2.4	11 (—) 2 (+) 2 (++)
Group 3, 8 Wks				
DAF only	9/14	232/203	18.0±1.8	1 (—) 2 (+) 2 (++) 4 (+++)
DAF + Smoke	14/14	237/218	20.0±2.0	3 (—) 6 (+) 5 (++)

* Gross observations were made at the time of sacrifice. Increasing precancerous damage is indicated by an increasing number of pluses.

diet for fourteen weeks, and received the smoke from twenty cigarettes hourly for eight hours each day. From the data, it can be seen that depletion of the riboflavin in the liver by the carcinogen was inhibited, and gross estimation of the degree of precancerous cirrhosis substantiated this finding. Note that seven of the ten smoked animals sacrificed had normal appearing livers, while the livers from the animals not receiving smoke were considerably damaged. In addition, the mortality was high in those animals not receiving smoke, while all the rats receiving smoke survived.

In subsequent series, less smoke (five cigarettes per hour) was used due to the obvious strain on the animals receiving smoke from twenty cigarettes per hour. Again, the same findings were

observed: animals receiving smoke and DAF had reduced liver damage, their riboflavin values were higher than those animals not receiving smoke, but receiving only the carcinogen. Considering the number of rats used, it is felt that the higher riboflavin values of the smoked animals represented an inhibition of a precancerous biochemical change characteristic of this carcinogenic process.

Intraperitoneal injections of the crude cigarette smoke tar every other day produced the same type of inhibition as smoke. In group 1, Table 3, eight of the tar-injected rats had normal

TABLE 3

Effect of Crude Tobacco Tar on Liver Riboflavin and the Development of the Precancerous State in Rats Fed 2-Diacetylaminofluorene

Experiment	No. Survived/ No. Started	Wt. at Sacrifice/ Wt. at Start	Riboflavin Microgm/gm.	No. of Rats Showing Gross Damage
Group 1				
8 Wks DAF + Oil	9/12	230/257	18.7±1.0	1 (—) 1 (+) 7 (+++)
8 Wks DAF + Tar	13/13	250/239	20.9±1.7	8 (—) 2 (+) 1 (++) 2 (+++)
Group 2				2 (+) 5 (++) 2 (+++)
8 Wks DAF + Tar*	11/13	256/211	20.4±1.2	7 (—) 1 (+)
12 Wks DAF + Oil	3/3	237/222	14.7±2.0	2 (++) 1 (+++)
12 Wks DAF + Tar	5/5	250/211	15.8±0.6	1 (—) 4 (+)

* Three of these animals were not sacrificed.

livers, while only one of the livers of the peanut oil-injected animals was normal. The riboflavin values again showed a higher concentration in the tar-injected animals, and the concentration was about the same as found for smoked animals. This experiment was repeated in group 2, Table 3, and a similar result noted.

It was of interest to determine the approximate period of DAF feeding which produced the first difference in riboflavin between tar-injected rats and controls. In Table 4, the results of feeding

TABLE 4

Effect of Short-Term Feeding of DAF and Concurrent Injections of Tar

Experiment	No. Survived/ No. Started	Riboflavin Microgm/gm.	No. of Rats Showing Gross Damage
Group 1			
10 days DAF + Oil	3/3	25.9±2.6	3 (—)
10 days DAF + Tar	3/3	24.4±2.0	3 (—)
30 days DAF + Oil	3/3	19.1±3.3	3 (—)
30 days DAF + Tar	3/3	24.6±2.3	3 (—)

from 10 to 30 days can be seen. By the end of thirty days, liver riboflavin had dropped in the DAF-fed animals but was maintained at close to basal levels in the tar-injected animals. Thereafter (8 and 12 weeks, Table 3), there remained a difference between controls and tar-injected rats, even though both values were decreasing. It is to be noted throughout these studies that the smoke and tar-injected animals receiving DAF had a much lower ratio of mortality than the animals fed DAF alone. We feel that this represents further evidence for the inhibitory effect of tobacco tar or smoke against the development of the precancerous state.

The effect of this tar fraction was extended to azo dye liver carcinogenesis. Male rats fed diets containing .06 per cent 3′MeDAB were injected with the crude tar fraction as described above. These animals were sacrificed after either 8 or 14 weeks and the extent of liver damage was noted. The control and the tar treated groups were almost identical with respect to concentration of liver riboflavin (Table 5). However, the tar treated

TABLE 5

Effect of Tobacco Tar on Liver Riboflavin and the Development of the Precancerous State in Rats Fed 3′Methyl-4-Dimethylaminoazobenezene

	Wt. at Sacrifice/ Wt. at Start	Riboflavin g/gm. Liver	Gross Damage
9 Wk. Group			
3′MeDAB + Oil*	277/316	16.8±1.3	+++
3′MeDAB + Tar	317/330	16.4±1.1	—
			+
14 Wk. Group			
3′MeDAB + Oil*	352/370	17.9±2.6	+++
3′MeDAB + Tar	362/397	16.1+1.4	+

* These control animals were injected (I.P.) every other day with 0.1 ml. peanut oil, the vehicle used for the tar injections.

animals grossly showed considerably less damage than the control rats. These findings are again indicative of an inhibiting action of a cigarette smoke tar fraction upon carcinogenesis.

That the inhibitory effect was not due to a non-specific injection of a suspension, *i.e.*, due to purely physical reasons, was suggested by further experiments. Washed norit suspended in peanut oil was administered to rats maintained on a diet containing DAF. The dosage was identical to that of the tar-injected animals and was also administered intraperitoneally. The data on liver riboflavin, survival rate, and gross examination of the liver condition indicated that the injected suspension of norit offered no protective action.

The crude tar used up to this point was that prepared in our laboratory and may not be identical with tar obtained under physiological conditions. In other words, no specific information is available as to how closely the tar fraction employed resembled the tars produced during the actual smoking of cigarettes. When a commercial tar fraction became available, which presumably was identical to that obtained under physiological conditions, additional studies were continued with this new tar preparation.

From the results shown in Table 6, it is concluded that feeding or painting of the commercially produced tobacco tar fraction had no effect upon 3'MeDAB or DAF-induced liver tumors. These results are at variance with those of Mori *et al.* (20)

TABLE 6

The Effect of Cigarette Smoke Condensate on Cancer Induction by Administration of Either 3'Methyl-4-Dimethylaminoazobenzene or Diacetylaminofluorene for 14 Weeks*

Group No.	Treatment	No. of Animals	Wt. at Sacrifice/ Wt. at Start	Gross Liver Damage	Final Tumor Incidence
1	3'MeDAB — Controls	30	235/211 g.	+++	16/27
2	3'MeDAB + tobacco smoke tar painted on neck (2 times weekly)	15	232/231	+++	9/14
3	DAF	11	199/220	+++	5/10
4	DAF + tar in diet	11	224/215	+++	4/9
5	DAF	25		++++	18/22
6	DAF + tar painted on neck (2 times weekly)	25		+++	15/22

* Obtained from: Eusta Paper Corporation, Pisgah Forest, North Carolina.

wherein the incidence of azo dye-induced liver tumors was reportedly decreased by feeding of cigarette smoke tar fractions. It is not improbable that different smoke tar fractions would vary considerably in composition depending upon exact conditions of combustion and other factors.

That feeding the tar or painting it on the skin would produce results different from intraperitoneal injections is not surprising. The route of administration, from the viewpoint of concentration alone, may be of tremendous influence on the final result. It can only be concluded that this tar fraction, administered by painting or by incorporation into the diet, did not alter the final tumor incidence in rats fed DAF or 3'MeDAB.

A final investigation was made to measure the effect, if any, of the cigarette smoke tar condensate upon the oxidative destruction of azo dye by liver homogenates. The smoke condensate was that prepared under physiological conditions. Miller and associates (15, 16) have shown that normal rat liver has the capacity to destroy 4-dimethylaminoazobenzene. Also, they have shown that the intraperitoneal administration of methlycholanthrene (MC) 2-3 days prior to sacrifice will greatly increase the destruction of the added azo dye in the liver homogenate system. Employing the same procedure as that described by Miller, we carried out studies as follows:

Group A	*Liver System*	*Results*
Female weanling rats injected (IP) with 10 mg. methylcholanthrene (MC) in 0.5 ml. propylene glycol. Sacrifice after 3 days.	0.5 ml. of 10 per cent homogenate (3'MeDAB substrate).	11 γ dye destroyed
Controls—propylene glycol only	same	6 γ dye destroyed
Group B		
Male weanling rats injected 10 mg. MC as above.	same as above	16 γ dye destroyed
Controls	same as above	8 γ dye destroyed
Group C		
1. Male weanling rats injected I.P. with 5 mg. MC Sacrificed 3 days later.	0.4 ml. of 10 per cent homogenate (DAB) substrate)	19 γ dye destroyed
2. Same but injected with smoke condensate 0.2 ml. in propylene glycol.	same	12 γ dye destroyed
3. Controls.	same	destroyed 10 γ dye

From these findings we observed that as expected, the injection of methylcholanthrene did result in a greater destruction of azo dye by the liver homogenate system. However, the extent of this destruction did not equal that reported by the Millers. Under the conditions employed administration of the tar fraction did not result in any significant increase in dye destruction above that noted in the controls. This could possibly indicate that the tar fraction did not contain appreciable quantities of carcinogenic hydrocarbons. This test was probably not sufficiently sensitive to detect the small quantities of hydrocarbons that may be present in this smoke tar condensate. Finally, we tried to extract the smoke tar with suitable solvents which would remove carcinogenic hydrocarbons. This extract was concentrated and injected into weanling rats. Again there was no definite indication of any effect upon dye destruction by the liver system. With this final study we concluded that carcinogenic hydrocarbons were not present in this smoke condensate in significant quantities to be detected by this method.

Discussion

The authors are aware that obtaining a standard sample of test material is one of the difficulties in studying the effects of cigarette smoke on experimental animals; that is, the condensate of smoke or the tar itself. In the above experiments, two different preparations have been used, although just what the differences are between them is not known.

In spite of the above question, certain facts appear clear. The experiments which utilized smoke itself as well as crude tar indicated a delaying action by the smoke on the development of the precancerous state in rat liver. This inhibition of the development of the precancerous state was observed in gross pathological studies, concentration of liver riboflavin, and survival rate. Further, the growth of the animals was noticeably enhanced when the animals were administered crude tar. In each case where tar was given, weight gains during the testing period were greater than animals receiving DAF alone. In pharmacological investigations, growth alone is frequently the most sensitive measure of toxicity, and in this case appears to reflect the protective action of tar against the toxicity of DAF. Whether the toxicity is related

to the tumor-inducing properties of DAF or is a distinct property is not known, and may be a separate phenomenon.

Precisely what significance liver riboflavin bears to the carcinogenic process is not known, but the analyses may indicate the extent of normal liver tissue remaining at any selected time. If this be the case, then smoke appeared to protect the liver against the effects of the carcinogen DAF.

When the azo dye was fed, tumors developed readily not only in controls, but in tar-injected rats as well. No protective effect was seen in the riboflavin determinations although there appeared to be a visible difference in gross pathological specimens. This was true whether tar was fed: with the diet, injected, or painted on the neck. Additional evidence that cigarette tar has no influence on 3′MeDAB tumor induction was obtained from the *in vitro* tests, in which cigarette tar condensate did not have the capacity to increase enzymatic destruction of MDAB that was seen for methylcholanthrene. It may logically be assumed that the differences between azo-dye tumor induction and DAF tumor induction are involved here.

The mechanism of the inhibition of tobacco tar can only remain a subject for speculation at this time. Miller, McDonald, and Miller (18) have found a similar inhibition of 2-acetylaminofluorene carcinogenesis by methylcholanthrene. Their data on mortality and delay in tumor development are similar to the results of this investigation. Inhibitory action of methylcholanthrene against the azo-dye carcinogens has been carefully studied and verified in several laboratories (14, 16, 22, 23), and an explanation offered on enzymatic terms (22). Other experiments on antagonism of 2-acetylaminofluorene by various carcinogens (10, 18, 19, 20), and the use of methylcholanthrene to inhibit carcinogenesis caused by agents other than azo dyes or DAF (11, 24, 27, 28) well substantiate early ideas on carcinogenic antagonism by Lacassange and co-workers (12). Evidence indicating the presence or absence of carcinogens in cancer-producing quantities in cigarette smoke has been conflicting and will not be considered. However, one would be amiss to deny the presence of some carcinogenic hydrocarbon in specially-prepared tars from cigarette smoke.

An alternate explanation of the inhibition is the presence of non-carcinogenic antagonists to DAF in tobacco tar. Numerous

examples of non-carcinogenic inhibitors exist in the literature (reviewed by Crabtree, 4), and can be found to exist in such diverse structures as *p*-hydroxypropiophenone (25) and riboflavin (8). Thus, a rational explanation to the inhibition of DAF carcinogenesis would not have to depend on the presence of carcinogenic substances in tobacco smoke and tar.

Summary

1. The effect of 2-diacetylaminofluorene feeding on liver riboflavin and deoxyribose nucleic acid was studied. It was found that hepatic riboflavin is decreased during the feeding of DAF, and that DNA concentrations remain constant during the time periods studied.

2. Cigarette smoke and crude tar collected from smoke when injected I.P. into rats delay the appearance of the precancerous state in animals fed DAF, as seen in gross damage of the liver and delay in riboflavin lowering of precancerous liver.

3. No protective action of tobacco tar was observed when the carcinogenic diet was composed of the azo dye and liver carcinogen 3′-methyl-*p*-dimethylaminoazobenzene. Inhibition was attempted by feeding and injecting the tar, by applying it to the skin, and by studying the effect of tar on the enzyme system responsible for destroying the azo dye.

4. Administration of a commercially prepared condensate of cigarette smoke to animals fed diets containing either 3′methyl-4-dimethylaminoazobenzene or 2-diacetylaminofluorene did not affect the rate of cancer induction or the final tumor incidence. It must be recognized that different smoke tar fractions vary considerably in composition depending upon the conditions of combustion and other factors.

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ON TRANSPLACENTAL INFECTION WITH COXSACKIE VIRUS

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The problem of the origin of fetal malformations is extremely important from the social point of view and has been widely discussed in recent years. It was one of the principal subjects of papers during the last International Congress of Pediatrics in Copenhagen.

The main interest has been centered around immunological and infectious mechanisms as the origin of malformations. In case of the former, the serological conflict due to the Rh factor is placed in the foreground (16, 17, 18), whereas considering infectious origin, reference is made to pregnant women infected with *Treponema pallidum* (27, 24), or to the protozoan *Toxoplasma gondii* (21). Among virus diseases, the most thoroughly investigated cases up till now were infections with German measles (12–14).

According to the data of Gregg (12, 13), 83 per cent of malformations in the embryo occurred when the mothers were sick during the first three months of pregnancy, and 61 per cent when the mother was in the fourth month of pregnancy. American statistics (19, 26) show lower percentages. However, the Australian data correspond more fully to the actual conditions (they were collected in older children, where greater emphasis was placed upon less perceptible characteristics, like deafness or secondary muteness). In these investigations the deafness occurred in 75 per cent of children with malformations, microcephaly in 70 per cent, eye disturbances, particularly cataract, in 66 per cent, developmental malformations of the heart in 63 per cent and changes in the teeth in 45 per cent. Furthermore, the Australian authors recorded frequent abortions and premature births in women who had German measles.

Experiments by Töndury (36) showed a *phasic* specific sensitivity of the embryo to rubella infection with preference of organs of ectodermal origin, particularly epithelium.

The papers by Gregg *et al.* (12, 13) and of Swan *et al.* (34)

were the first reports on the subject of the origin of malformations caused by transfer of a virus from mother to embryo. Recently, more papers discussed the problem of virus infection of the embryo through the placenta. A summary of the relation between malformation of children and infectious diseases of the mother during pregnancy were published by Swan in 1949 (33) and by Francois and Verriest in 1955 (9). The latter discussed primarily the problem of *rubella* and emphasized that other virus diseases: *chickenpox*, *poliomyelitis*, *hepatitis epidemica* and *influenza*, also can produce malformations.

It is interesting to note that infectious disease of the embryo need not always be concurrent with the sickness of the mother. Virus infection of the embryo can lead to the origin of malformation even during latent infection of the mother. If transplacental infection occurs early in pregnancy, profound damage and abnormality result. Such damage can lead to death of the embryo, or, in case it lives, organs develop abnormally. Virus infections in the earlier stages of pregnancy can lead to malformations, but if they occur in later stages of pregnancy, they produce a virus disease of the older embryo and of the newborn. The disease may occur after birth in a period shorter than the accepted incubation period: in these cases it is assumed that the infection took place in the uterus.

It has been shown that the virus can pass through the placenta from mother to embryo or fetus. For instance Lynch (23) describes a case of a dead embryo resulting from intra-uterine *vaccinia* infection by the virus with which the mother was vaccinated. Blank and Rake (5) describe cases of pox marks in children as a result of vaccination of the mothers. The transfer of herpes virus from mother to fetus was described by Zuelzer and Stulberg (40). They showed that prematurely born babies are particularly sensitive and that they may be infected, during birth. There occurs probably a general virus infection with necrotic damage in the liver and other organs. The disease generally has a lethal outcome. Similarly, Quilligan and Wilson (28) observed a recently born baby with herpetic infection, encephalitis and liver necrosis. Nine days before the birth the mother became sick with *gingivostomatitis herpetica*. Lutzenkirchen (22) described a case of *eczema herpeticum* in the first three months of pregnancy which was cured within three weeks. Similar cases of infection

with herpetic virus (herpes zoster) were observed by Swan (30-33), Freud (10), Bohn (6), Kalb (20), and others. The herpetic infection is relatively easy to determine in virologic investigations: in these cases, the presence of the virus was demonstrated in the liver, mouth and throat, in the pharynx, stomach, lungs, adrenals, spleen, and brain. Another virus disease in newborns, and babies having a generalized course, is cytomegaly or infection of salivary gland (Germer, 11). It is a mortal disease. The fetus can be infected through the placenta as shown by Bellamy (3). Clinically this disease can resemble the embryonic infection with herpes virus, erythroblastosis, toxoplasmosis. In older children and in adults the disease occurs as an interstitial pneumonia (Wyatt 37, 38, 39).

Bowers and Danforth (1953) and Bates (1955) described cases of transplacental infection by polio viruses. Poliomyelitis is rarely seen in fetuses. Anderson *et al.* (1) mention 53 cases of poliomyelitis in mothers and only 2 babies showed malformations. In one of them paralysis of the eyelid, in the other, constriction of the pylorus was the outstanding symptom. Probably the virus infects the fetus during the viremic stage in the mother, as was pointed by Shaeffer and collaborators (1954). They have isolated polio virus from the fetus and from the placenta in a case of a miscarriage, which occurred on the eleventh day after the beginning of poliomyelitis in the mother.

Transplacental infection by Coxsackie virus (group B) was observed in the maternity wards in Johannesburg by Montgomery and associates (1955), who described 13 cases of myocarditis in newborns. Seven of them died. Flamm (1955), and Berger and Roulet (1952) showed in pregnant mice that Coxsackie virus can pass through the placenta.

In 1954 we had the opportunity to observe a case with fetal malformations in which Coxsackie virus type A_4 can be regarded as the etiologic agent.

Case History

Mrs. A. J. (case history 2298/54) was admitted to the maternity ward in 32nd week of pregnancy because of premature labor pains. It was her first pregnancy. She felt quite well during this time except for a short period of fever (100.4° F.) in the first month. Influenza was diagnosed. Familial history was unimportant. There were no fetal malformations in the families of the parturient or her husband.

Because of a worsening pulse of the fetus the parturition was ended by forceps. The newborn was a girl. Her general aspect was good, the weight 1800 gr., the length of the body 44 cm., the circumference of the head 34 cm. The head was large, the right eyeball underdeveloped, the right ear malformed, consisting of three small lobes without cartilages; the right external aureal meatus junctions very small on both cheeks papillomata.

Further observation of the baby led to the conclusion that there were malformations in the alimentary tract and the upper respiratory tract. Narrowing of the esophagus and an esophago-tracheal fistula were diagnosed. In forced feeding, the tube could be introduced only to the depth of 11 cm. and the introduced fluid was returned by mouth and nose with simultaneous cyanosis. In the first day of life a small quantity of meconium was delivered by the child. On the fourth day the baby died with the symptoms of an aspiration pneumonia.

The autopsy showed: 4-day old baby with numerous congenital malformations. Cause of death: diffuse bilateral bronchopneumonia.

Additional examinations: Blood groups: mother, O, Rh+, no immune antibodies; father, O, Rh−; baby, O, Rh+; Coomb's reaction-negative; Wassermann reaction with the bloods of the mother and of the child—negative.

Numerous malformations together with the case history made plausible the supposition of an intrauterine damage of the fetus by toxoplasmic infection. Therefore blood, meconium and cerebrospinal fluid of the baby were sent for examination to Hirszfeld Institute of Immunology and Experimental Therapy, University of Wroclaw, Poland.

Examinations for Toxoplasmosis

In mice injected with pathologic material, no toxoplasma was found. Serologic examination of mother's serum by the method of Sabin and Feldman (Dr. Kozar, Institute of Marine and Tropical Medicine) was also negative.

Neurologic Examination

Intracerebral and intraperitoneal injections of cerebrospinal fluid of the baby into adult mice did not cause any pathogenic symptoms. The same was also true after similar injection of 48 hour old mice. Quite different were the results of injection of the suspension of meconium into baby mice. A virus of the Coxsackie group was isolated and it was shown that it belongs to group A, type 4.

Forty-eight hour old baby mice were injected with 10 per cent suspension of meconium in physiologic saline with 1000 units of Penicillin and 500 mg. streptomycin per 1 ml. After 20 days the first baby mouse died, and after 3 days another two were dead. All of them had paralysis of the limbs. The next few baby mice were in-

jected with the suspension of the thorax muscles of the first passage mice. All died with the symptoms of paralysis. In the following passages the incubation period was constantly 48 hours.

In table 1 the results of virologic examinations of the cerebrospinal fluid and of meconium are given, as well as the length of the incubation period.

TABLE 1

Isolation of a Coxsackie Virus Strain From the Meconium of a Newborn Infant with Malformations

Material	1st Passage	2nd Passage
Cerebrospinal fluid	0/6	...
Meconium	3/10	8/8

	The Day of Death											
	1	2	3	4	5	6	1	2	3	4	5	6
	..	1/10	..	..	2/10	..	..	8/8	..	..	..	..

In the numerator of the number of dead animals is indicated, in the denominator—the number of injected animals.

The presence of the virus in diseased animals was also established in internal organs, *i.e.*, in the liver, spleen, brain and in the contents of the intestines.

Adult mice were injected twice with a suspension from the paralyzed muscles. There were no signs of disease, even if enormous doses of the virus were used.

The concentration of the virus in baby mice was very large—the maximum titer was about 10^{16} LD_{50}.

The neutralization reaction was made by using immune mice sera, which were obtained by injecting twice 2-2½ month old mice with suspension of the muscles of infected baby mice. The first injection was made intramuscularly, the muscle suspension being prepared with a mixture of paraffin oil and lanolin. The second injection was given subcutaneously, and the muscle tissue was suspended in physiological saline. The interval between injections was 4 weeks. The mice were bled on the 8th day after the second injection.

The Coxsackie strains A_1-A_{10} and B_1-B_4 was sent to us from Stockholm, Sweden, by Dr. Johnson and Dr. Heller.

The neutralization test was performed at room temperature during 1 hour. The volumes of virus suspension and serum so-

lutions were identical. The results of neutralization tests with antisera against types A_1-A_4 are summarized in table 2.

The neutralization test of strain A. J. by the serum of the mother of the baby A. J. is summarized in table 3. It ran as far as No. 1/50 dilution of the serum, when 5000 LD_{50} were neutralized.

TABLE 2

Neutralization of the Coxsackie Strain A. J. by the Anti-A_1-Anti-A_4 Sera

Sera	Dilution of the sera 1/50	1/100	1/250
Anti-A_1	4/4	4/4	4/4
Anti-A_2	5/5	5/5	5/5
Anti-A_3	5/5	5/5	5/5
Anti-A_4	0/5	0/5	0/5
Control (normal mouse sera)	4/4	4/4	4/4

The dose of the virus was 0.03 ml. of the dilution 10–12, *i.e.*, 10,000 DL_{50}. In the numerator is the number of dead animals and in the denominator the number of infected animals.

TABLE 3

Neutralization of Coxsackie Strain A. J. by the Serum of the Mother of the Baby A. J.

Sera	Dilutions of the sera 1/5	1/10	1/50
Mother of A. J.	0/5	0/5	2/4
Normal serum (control)	4/5	5/5	5/5

Note: The dose of the virus as in table 2.

Histopathologic examination of the infected baby mice showed changes in the thoracic and head muscle typical for Coxsackie virus infection (Dr. Kawecki).

The results indicate a successful isolation of A_4 Coxsackie virus from meconium of a newborn child with congenital malformations. At the same time presence of neutralizing antibodies for this particular type of virus were found in mother's serum. In view of frequent reports concerning the development of congenital malformations due to viral infections of the pregnant women, the occurrence of Coxsackie factor in such cases must be seriously taken into account. It is especially important because of wide prevalence of these viruses in the human population.

Summary

The authors describe a case of a newborn infant, with a series of developmental defects, who died 4 days after the delivery. The immediate cause of death was bronchopneumonia (oesophagotracheal fistula).

There were no indications of a serologic conflict between mother and child or an infection by *Toxoplasma gondii* as possible causes of developmental defects. From the child's meconium Coxsackie virus, type A_4 was isolated. In the cerebrospinal fluid the virus was not present. Mother's serum neutralized the isolated virus in a high titer.

In establishing the etiological relationships of developmental defects in newborn children the possible role of Coxsackie viruses should be kept in mind.

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INFRARED SPECTRA OF ACETONE EXTRACTS OF SUSCEPTIBLE AND INSECTICIDE RESISTANT STRAINS OF HOUSE FLIES[a, b]

DON W. MICKS AND K. R. P. SINGH

During recent years, the problem of insecticide resistance in insects has greatly increased. Consequently, more and more attention has been directed toward differentiating between susceptible and insecticide-resistant strains of insects in order to better understand the phenomenon of resistance. With its rapid development of tolerance to various chemicals, the house fly has been one of the most frequently used insects for such studies. Both morphological (March and Lewallen, 1950; Bigelow and LeRoux, 1954) and biochemical (Perry *et al.*, 1953; Sternburg *et al.*, 1954; March and Lewallen, 1956) comparisons of various strains have been made. As yet, there seems to be no simple, accurate method for detecting insecticide resistance in an insect population at an early level of development.

Since infrared absorption spectra of extracts have been successfully used to distinguish between strains and species of mosquitoes (Micks and Benedict, 1953; Micks and Scrollini, 1954; Micks and Partridge, 1956), it was decided to apply the infrared method to a study of extracts of susceptible and insecticide-resistant strains of house flies. It was the basic aim of this investigation to determine whether insecticide resistance *per se* is reflected at some particular point in the infrared spectra of house fly extracts.

Materials and Methods

Five separate batches (each representing a different generation) of seven strains of house flies, *Musca domestica* L., were used in these studies:

1. *NAIDM*—a susceptible laboratory strain obtained in 1948 as a combination of the Orlando and Beltsville strains.
2. *Dilan 45*—a resistant strain derived from the NAIDM-48 strain by selection with Dilan.
3. *Lindane 45*—a resistant strain originally secured from the USDA Laboratory, Orlando, Florida, where it was designated as their LLD strain.
4. *Dieldrin 45*—a resistant strain obtained from the NAIDM-48 strain by selection with dieldrin.

5. *Roberds*—originally obtained in the field, this laboratory strain is resistant to DDT and dieldrin.
6. *Bethesda 45 Malathion*—a resistant strain originally obtained from the field and now maintained as a laboratory strain.
7. *DDT 45*—a resistant strain initiated by selection pressure against the NAIDM-48 strain.

Newly emerged adults were collected and kept in the deep freeze until used. The sexes were separated and only female flies were employed in these studies. The specimens were weighed in batches of 50 mgm. each and homogenized in a motor-powered tissue grinder with 1 ml. of acetone for ten minutes. The material was then transferred to centrifuge tubes and centrifuged for fifteen minutes. Then 0.4 ml. of the supernatant was mixed with 250 mgm. of KBr of infrared quality, using a mortar and pestle. The mixture was dried under vacuum for twenty-four hours. The material was made into pellets with a thickness of 0.031 cm. using a Baird die. The die was placed in a Carver press at 10,000 lbs. for ten minutes. The resulting pellet was then ready for analysis. All infrared absorption spectra (2–16 microns) were determined with a Baird doublebeam recording instrument equipped with a sodium chloride prism, except for the curves shown in Figure 1 which were obtained with a Beckman single-beam recording spectrophometer. Infrared curves were recorded from several pellets prepared from each of the five batches of each strain.

Results

A comparison was first made of the different fractions obtained during the extraction procedures in order that identification of some of the chemical constituents might be made. This information is necessary for interpretation of differences in curves and to ascertain which materials are responsible for the changes in the spectra. Figure I compares curves obtained from (1) whole dried flies, (2) the extract from extraction of fly tissues with distilled water, (3) the residue from the above extraction, (4) an acetone extract and (5) the residue from acetone extraction. It can be seen that extraction with distilled water intensified the hump at 6.8 μ in the extract and markedly increased absorption of the band with a maximum at 7.15 μ. The spectrum obtained from the residue was essentially like that representing whole dried specimens. Upon acetone extraction, however, an entirely different type of curve resulted when the extract was analyzed. First of all, there was an attenuation of C-H groups at 3.4–3.5 μ and the ester carbonyl groups at 5.8 μ disappeared. As judged by the acetone residue spectrum, practically all the lipid esters at 5.8 μ were removed. The absorption pattern from

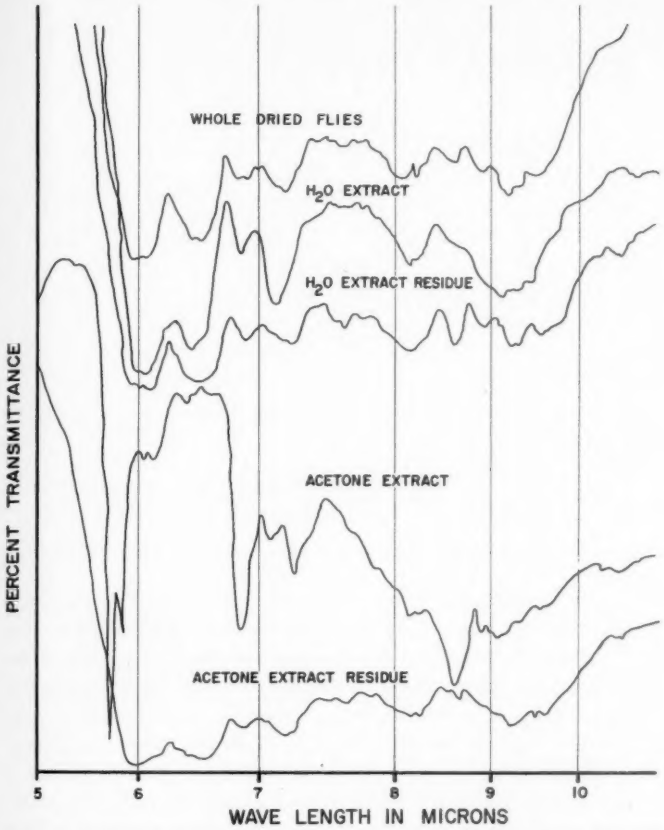

FIG. I. Infrared absorption spectra of whole flies and fractions obtained following extraction with distilled water and acetone.

8.4 μ to 8.82 was also changed by the extraction of lipids. The acetone extract residue curve was very similar to that obtained from dried flies.

Since the infrared curves obtained from acetone extracts appeared to show the greatest promise for purposes of differentiation, the next step was to ascertain the degree of reproducibility of the spectra characterizing a given strain. Figure II shows the curves obtained from extracts of five generations of the Dilan

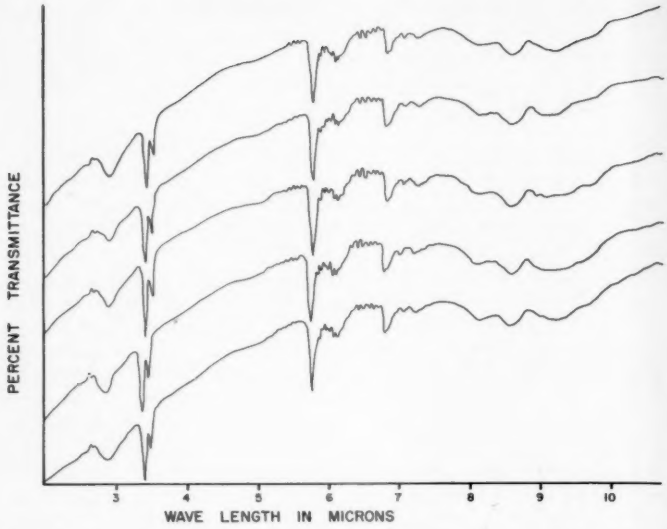

FIG. II. Spectra of acetone extracts of five generations of the Dilan 45 strain.

45 strain. It will be noted that the absorption spectra are essentially identical. On this basis, a comparison was made of the lipid extract curves of all seven strains of flies (Fig. III). It became apparent that the most informative range of the spectrum was from approximately 2 μ to 10 μ. There are minor differences between extracts of the different strains in the relative depths of the C-H stretching band at 3.4 μ, the band due to ester carbonyl groups at 5.85 μ and the band representing CH_2 and CH_3 bending groups at 6.8 μ. However, maximum differentiation could be obtained by comparing the shape and depth of the shallow band from 2.7 μ to about 3.1 μ and the shape of the broad band from 7.8 μ to 10 μ, particularly that portion with a maximum at 8.6 μ.

It was found that the band starting at 2.7 μ was more shallow in spectra of the Dieldrin, DDT and Lindane strains. The band with the absorption maximum at 8.6 μ was distinctly deeper in the DDT, Roberds, Malathion and Lindane strains than in the remaining three. In general, the infrared curves representing the NAIDM and Dilan strains showed a greater similarity than any other two spectra.

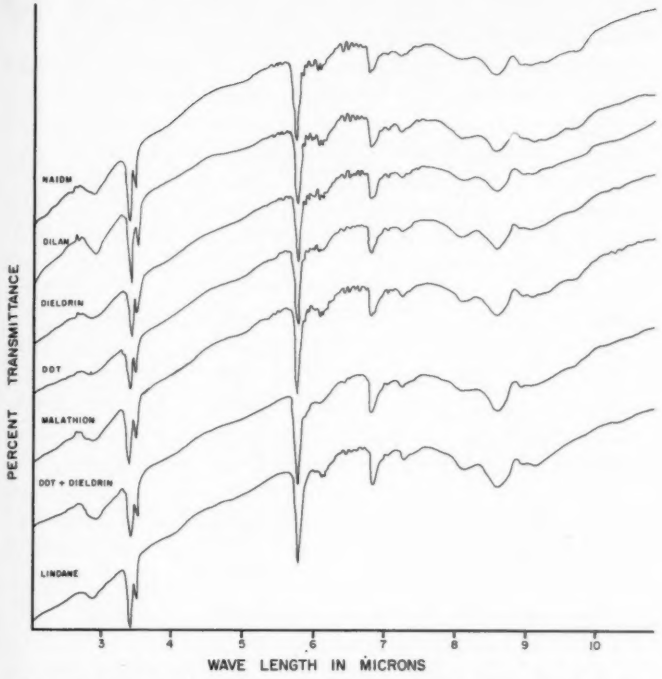

FIG. III. Characteristic absorption spectra of acetone extracts of the seven strains of house flies.

Discussion

A comparison of the curves obtained from the acetone residues and extracts suggests that one of the identifying characteristics of the strains, *i.e.* the absorption at 8.6 μ is partly due to their lipid content. It is interesting to note that these particular strain differences must be associated with specific lipid fractions only, since there were no differences between strains as to their total fat content on the basis of the conventional Soxhlet extraction procedure.

There is no apparent explanation for the great similarity between the NAIDM (insecticide-susceptible) and Dilan (insecticide-resistant) strains as judged by their infrared spectra except for the fact that the latter strain was derived from the NAIDM

strain. Furthermore, there is no indication that the strain differences observed are linked with resistance *per se*.

Prior to this work, paper chromatographic methods were used to compare the strains herein studied. Several sets of one-dimensional chromatograms of aqueous extracts revealed no significant differences in the patterns of free amino acids present with the exception that the total amino acid content was consistently lower in the Malathion strain than any of the others. At this point it was decided that the infrared method showed much greater promise as a differentiating tool since it measures biological systems at the intramolecular level.

Although the spectral differences between acetone extracts of the strains studied are somewhat subtle, they are nevertheless highly reproducible and therefore are interpreted as true strain differences. Numerous other investigators have studied biological systems with infrared methods and have likewise found that differences in the absorption characteristics of similar material are not great. For example, O'Connor *et al.* (1956) reported that identifications of microogranisms will have to be based on very small differences, often not much above the reproducibility with which the spectra can be measured. Certainly if the characteristics which may differentiate strains belonging to a single insect species are those substances which are present in small concentrations, further efforts must be made to separate the identifying portion from the large mass of material common to all strains, which may mask important vibrational regions.

It is planned, therefore, to extend these studies along similar lines utilizing other fractionation procedures, particularly concentrating upon the purification of residues. For example, it is known that insecticide resistance in insects has a genetic basis. It would thus appear worthwhile to extract nucleic acid fractions for analysis since maximum strain differences may be reflected in this particular component. Furthermore, DDT is known to be broken down in resistant house flies to two non-toxic products, DDA (dichloro-diphenyl-acetic acid) and DDE (dichloro-diphenyl-ethylene). These metabolites have not been found in measurable quantities in susceptible strains. Therefore, DDA and DDE, which have been obtained in pure form will also be studied by infrared techniques to determine at which points in the spec-

trum they exhibit characteristic absorption bands. Such information may lead to the detection of developing DDT-resistance at a fairly early stage.

Summary

Acetone extracts of seven strains of susceptible and insecticide-resistant house flies, *Musca domestica* L., were compared with infrared spectrophotometric techniques. All strains except NAIDM and Dilan could be differentiated by comparing the shape and depth of the shallow band from 2.7 μ to 3 μ and the shape and depth of the broad band from 7.8 μ to 10 μ, particularly that portion with a maximum at 8.6 μ. Differences between strains as to their absorption in this latter region appear to be due in part to their content of specific lipids since there were no measurable differences in total fat.

The results thus far obtained indicate that although certain strains of susceptible and insecticide-resistant house flies may be differentiated on the basis of subtle, reproducible differences in the infrared spectra obtained from their acetone extracts, these differences do not appear to be linked with resistance *per se*. Plans for extending this investigation are presented.

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ELECTROCARDIOGRAPHIC STUDIES IN EXPERIMENTAL CHAGAS' DISEASE IN MICE (MUS MUSCULUS)[a]

A. PACKCHANIAN AND A. ROBINSON[b]

Electrocardiographic changes in man suffering with Chagas' disease were first described by E. Chagas (1930) and subsequently confirmed by various investigators, *i.e.*, Mazza and Freire (1930); Dias, Laranja, and Nobrega (1945); de Soldati and Rabenko (1951); Rosenbaum and Alvarez (1955).

Electrocardiographic studies in experimental Chagas' disease in laboratory animals have been hindered in the past because of the lack of suitable animals. ECG studies on a limited number of dogs both naturally and experimentally infected with *Trypanosoma cruzi* have been described by a few investigators; Pellegrino (1946); Laranja, Pellegrino, and Dias (1949). Such studies have not been widely used, however, because of the relatively large size of the dogs and the expense involved.

There is ample evidence that myocardial damage in mice experimentally infected with *Tr. cruzi* show histopathological changes analogous to those in man and dogs suffering with the same disease, Packchanian (1939, 1940). Although the desirability of using mice because of their small size and inexpensiveness is obvious, in the past it was impossible to record the rapid heart beat of these animals with conventional ECG apparatus. However, the apparatus described by Rappaport and Rappaport (1943) and slightly modified by O'Bryant, Packchanian, Reimer, and Vadheim (1949) solved this problem and made such studies possible.

The object of the present communication is to report the electrocardiographic changes in experimental Chagas' disease in mice.

Methods and Materials

The electrocardiograph. The apparatus used in this investigation consisting of a modified string galvanometer electrocardiograph with a 3-stage resistance capacity coupled boosting amplifier and coupling unit has been de-

scribed in a previous communication by O'Bryant, Packchanian, Reimer, and Vadheim (1949). This apparatus was slightly modified by increasing the camera speed from 75 to 90 mm. per second by the use of suitable pulleys (Fig. I).

The animal board. A new animal board (Fig. II) replacing the one used in the previous study was constructed with a wooden block (20 x 4 x 7 cm.) fastened in the center of a flat board (25 x 9 x .5 cm.). Four holes 5 mm. in diameter were drilled through the width of the block 1.5 cm. above the platform. Two pairs of holes were drilled respectively 2 and 2.5 cm. and 10 and 10.5 cm. from the back end of the block. An additional hole was drilled in the center of the back end of the block 1 cm. above the platform. L-shaped metal rods 4 mm. in diameter with x-ray holder clips welded to each rod were inserted two from each side in alternate holes. The inside of the clips were filed smooth and the spring tension was adjusted to hold the animal comfortably. The four side clips hold the legs in place and the back one is

FIG. I. Electrocardiographic assembly: (A) modified Einthoven string galvanometer, (B) amplifier, (C) coupling unit, (D) patient cable leads, (E) mouse on animal board, (F) precordial lead and holder. (The copper wire screen cage is not included.)

for the tail. Binding posts were attached to three of the side clips for RA, LA and LL lead connections. Red, white and blue tinsel wires connected each post to a double pull, double throw knife switch (1 x ½ inch) fastened to a plastic box (6 x 6 x 1.5 cm.) on the right side of the block. Four brass binding posts connecting the DPDT switch to ECG machine were also fixed to the plastic box. To the front end of the block was fastened a chain with a paper clip at the loose end, and a nail was placed in the center top of the block for adjusting the chain to maintain vertical tension of the head of the animal.

Method of holding animal on board. The mouse was placed on the board with either the dorsal or ventral side up (Fig. II). The limbs and tail were

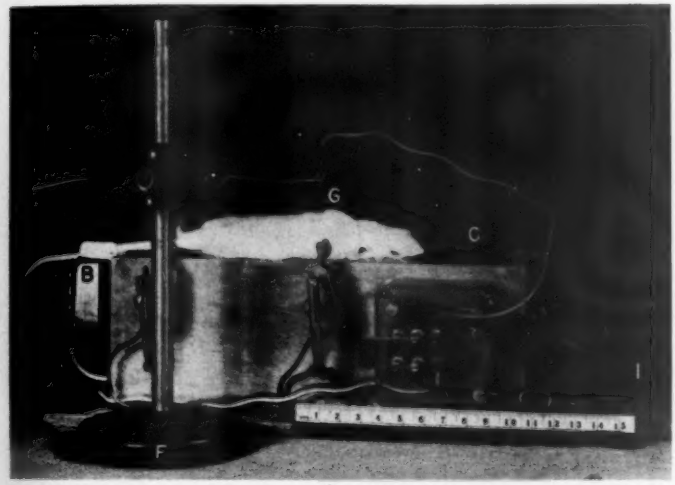

FIG. II. Animal board with mouse in supine position for precordial leads: (A) animal board, (B) clip holding tail, (C) chain holding head, (D) clip holding left hind leg and connecting electrode, (E) clip holding left fore leg, (F) stand to hold precordial lead, (G) precordial electrode, (H) switch DTDP in precordial position, (I) patient cable leads.

positioned by moving the clip holders so that they were held firmly but comfortably thus reducing muscle strain and tension. The head of the animal was held by attaching the paper clip to the upper incisors and the chain adjusted on the nail on top of the board to prevent the mouse from raising his head and introducing gross somatic tremor. The more comfortable prone position was used whenever the bipolar leads were taken. The animal was held in the supine position for precordial leads.

Animals used. A total of 81 white mice *(Mus musculus)* each weighing about 20 grams were used in this study. ECG tracings were made weekly or bi-weekly for one to five weeks on each of 58 mice in order to obtain a

normal pattern before 25 of this group were inoculated with *Trypanosoma cruzi* producing acute infection in 11 and subacute in 14. ECG studies were performed on the remaining 23 mice after a chronic infection of at least six months duration had been established in them.

Strains of Trypanosoma cruzi *used for infection.* Six strains of *Tr. cruzi* were used; all of which were infective to mice and produced a high percentage of myocarditis. Two of the strains, WBHT and Chilean, usually produce acute infection, terminating fatally within ten to fifteen days. The remaining four strains, *i.e.*, heart, Panama, Tatu, and Temple, produce subacute or chronic infections (Packchanian 1934, 1940, 1953; Packchanian and Sweets, 1947). All inoculations were made intraperitoneally. The inoculum for the first two strains was derived from blood of infected mice and for the remaining strains from culture media.

Methods of ascertaining the presence of infection in mice. The presence of *Tr. cruzi* infection was determined by direct microscopic examination of tail blood and xenodiagnosis with normal, laboratory-reared *Triatoma gerstaekeri* during the first two to eight weeks; and culture tests and histopathologic examination at the termination of the experiment. Acute infection was manifested by progressive increase in number of trypanosomes in the circulating blood until it was literally swarming with trypanosomes. The disease usually terminated fatally within 10 to 15 days. In the chronic infection, it was frequently difficult to demonstrate the scanty number of trypanosomes in the blood by microscopic search. At times even with diligent search no trypanosomes could be seen microscopically, however, positive xenodiagnosis and culture tests for *Tr. cruzi* at the termination of experiment confirmed the chronicity of the infection.

Methods of securing electrocardiograms. After the unanesthetized mouse was placed on the board, adequate contact was assured for the small electrodes by applying potassium chloride jelly. The animal, isolated from the table by the board's rubber supports, and the amplifier were shielded in a copper screen cage (34 x 18 x 17 inches) which was grounded in order to reduce 60 cycle AC current and other outside interference. Initially a few mice were etherized and others were injected intraperitoneally with mephenesin or dimethylane in amounts varying from .4–18 mg./kg. to minimize possible muscular tension. ECG studies on these animals were not as clear-cut as those on unanesthetized animals, therefore this procedure was discontinued.

The standard clinical bipolar limb leads were used in this study, plus the precordial leads which might be compared with V_1, V_3, and V_5 in human electrocardiograms and in some instances aVL, aVR, and aVF leads were made. In order to reduce the "indifferent electrode" tendency, only a limited area of the precordium was used. Tracings were taken on acutely infected mice at intervals of one to three days until the mouse was dead or the infection was one month old. At intervals of three weeks series of four electrocardiograms were made in the subacute and chronic infected animals.

After tracings were processed, they were optically enlarged with a system having a minimal curvilinear distortion to a scale in which 0.2 seconds was represented by 10 centimeters on the horizontal axis and 1 millivolt was

represented by 6.0 centimeters on the vertical axis. Measurements of time and amplitude of wave complexes were made from this enlarged scale. These were checked against measurements made from the original tracings with the aid of a magnifier and vernier caliper.

ECG data was tabulated on each of the four groups of mice on an individual animal basis. Means were calculated for values of R-R's, S in lead I; P, R, S, R′, T, P-R segment, QRS segment, Q-T interval, and heart rate in lead II, and R, S in lead III. Scatter graphs were made for each group from lead II to determine whether there was any correlation between the values of R to R′, of R to QRS, and of R′ to QRS. Because variations in leads I to III made measurement difficult, time values were not calculated on these leads. For the same reason, comparison of the diseased mice with the normal mice was based on the means obtained in the respective bipolar leads II.

For comparison, a few tracings were secured on a 2-channel cathode-ray oscilloscope and a modern high-speed two-channel hot-stylus electrocardiograph at a paper speed of 75 mm. per second.

Experimental Data

With the use of the modified Einthoven string galvanometer assembly at a camera speed of 90 mm. per second, well defined electrocardiograms were obtained from normal mice and mice infected with *Trypanosoma cruzi* (Figs. III, V, VI, VIII, IX).

Photographs of a few oscilloscopic tracings were compared with enlargements of string tracings for fidelity of string and found comparable (Fig. VII). Because of considerable variation in the mouse electrocardiograms from beat to beat, it was impossible to set up a "standing wave" on the oscilloscope. Tracings secured of normal mice with the modern high-speed two-channel hot-stylus ECG at paper speed of 75 mm. per second were deficient in reproducing curves showing accurate amplitude (Fig. IV).

The new animal board made manipulation of mice relatively easy with no edema or muscular tremor.

Normal mice injected intraperitoneally with mephenesin or dimethylane showed gross decrease in heart rate to as low as 200 beats per minute according to dosage but no spreading of the QRS segment. The P-R segment was increased as well as T-P segment. A similar change was noted with ether.

Eighty-nine ECG's taken from 33 normal mice, 63 with standard bipolar leads and 26 with precordial leads were clear-cut and readable. Differences discernable in the precordial and apical leads were also seen in bipolar leads. The Q-T and P-R intervals

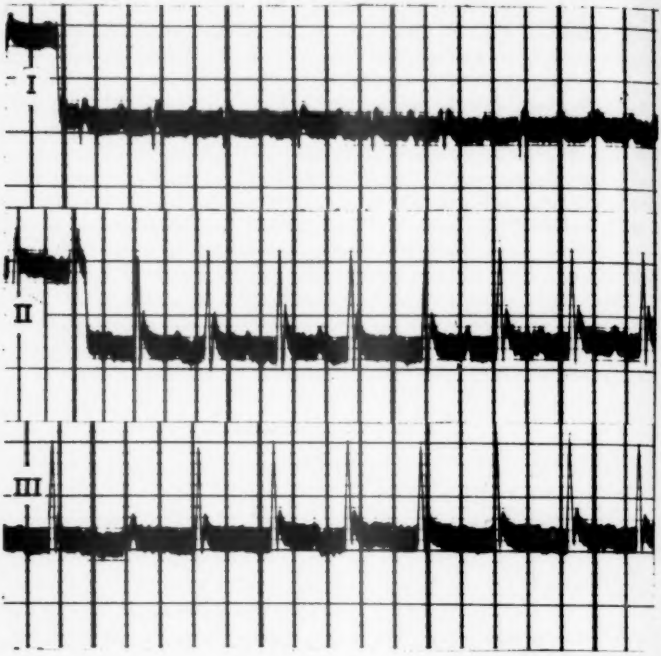

FIG. III A typical normal mouse electrocardiogram showing variations in P wave, average position of R, R′ and high T take-off.

and the QRS duration adjusted to the various ranges of heart rates tabulated for the leads II of 46 ECG's taken from 33 normal mice are shown on table 1. The means values of both amplitude and time factors calculated from single representative ECG's from the 33 normal mice are found on table 2. Statistical analysis of the findings of table 2 are shown on table 3.

Electrocardiographic studies of mice experimentally infected. One hundred and six ECG tracings secured with standard leads at various intervals from 48 mice infected with acute, subacute or chronic Chagas' disease were analyzed (Table 1). Out of this number, 48 tracings, one representing each mouse, were used in determining the mean values for each group (Table 2). Statistical analysis for significant findings in the infected groups from table 2 are shown on table 3.

TABLE 1

Q-T, P-R Intervals and QRS Duration in Lead II Versus Heart Rates on Normal Mice and Mice Infected with Trypanosoma Cruzi

Heart Rate Per Minute	Normal Mice Q-T Interval No. Mice	Lower	Upper	Means	Acute Infection Q-T Interval No. Mice	Lower	Upper	Means	Subacute Infection Q-T Interval No. Mice	Lower	Upper	Means	Chronic Infection Q-T Interval No. Mice	Lower	Upper	Means
500–550	3	0.045	0.050	0.048	1	0.055	0.055	0.055	1	0.050	0.050	0.050	3	0.040	0.045	0.043
551–600	5	0.042	0.045	0.044	10	0.040	0.062	0.051	2	0.045	0.052	0.049	5	0.024	0.040	0.037
601–650	6	0.034	0.052	0.044	6	0.045	0.052	0.049	10	0.035	0.047	0.039	8	0.036	0.045	0.040
651–675	12	0.036	0.050	0.041	8	0.040	0.055	0.048	16	0.030	0.052	0.039	7	0.027	0.047	0.036
676–700	9	0.030	0.052	0.043	4	0.040	0.047	0.043	9	0.029	0.042	0.039	4	0.035	0.045	0.039
701–725	8	0.034	0.040	0.037	2	0.037	0.040	0.039	7	0.033	0.039	0.037	1	0.033	0.033	0.033
726–780	3	0.035	0.045	0.039	0	....	....	....	1	0.040	0.040	0.040	0	....	....	....
		P-R Interval				P-R Interval				P-R Interval				P-R Interval		
500–550	3	0.040	0.050	0.045	1	0.040	0.040	0.040	1	0.040	0.040	0.040	3	0.040	0.050	0.043
551–600	5	0.035	0.040	0.037	10	0.035	0.045	0.040	2	0.043	0.045	0.044	5	0.032	0.043	0.040
601–650	6	0.035	0.044	0.040	6	0.035	0.040	0.037	10	0.037	0.045	0.040	8	0.036	0.055	0.042
651–675	12	0.030	0.045	0.035	8	0.034	0.040	0.037	16	0.035	0.045	0.039	7	0.036	0.046	0.041
676–700	9	0.033	0.045	0.039	4	0.035	0.038	0.036	9	0.027	0.045	0.040	4	0.033	0.050	0.039
701–725	8	0.033	0.042	0.037	2	0.035	0.037	0.036	7	0.037	0.042	0.039	1	0.038	0.038	0.038
726–780	3	0.035	0.038	0.036	0	....	....	....	1	0.035	0.035	0.035	0	....	....	....
		QRS Duration				QRS Duration				QRS Duration				QRS Duration		
500–550	3	0.027	0.035	0.031	1	0.035	0.035	0.035	1	0.033	0.033	0.033	3	0.019	0.022	0.020
551–600	5	0.020	0.025	0.023	10	0.024	0.038	0.032	2	0.027	0.030	0.029	5	0.016	0.025	0.021
601–650	6	0.018	0.027	0.023	6	0.024	0.035	0.030	10	0.017	0.025	0.021	8	0.017	0.027	0.021
651–675	12	0.018	0.030	0.023	8	0.022	0.035	0.029	16	0.016	0.028	0.022	7	0.015	0.025	0.021
676–700	9	0.020	0.030	0.026	4	0.024	0.029	0.026	9	0.017	0.026	0.022	4	0.017	0.030	0.022
701–725	8	0.016	0.027	0.022	2	0.019	0.027	0.023	7	0.019	0.027	0.024	1	0.016	0.016	0.016
726–780	3	0.017	0.030	0.022	0	....	....	....	1	0.025	0.025	0.025	0	....	....	....

On scatter graphs made of each group and normal controls, it was noted that there was no correlation between values of R to R′ in any group, nor was there any correlation between R or R′ to QRS segment in any of the groups, each observation seeming to vary independently of the others. These measurements also varied independently of the heart rate, the T-P segment varying with the heart rate in inverse correlation.

In comparison with the normals for the values of QRS segment and Q-T interval, the difference of the means in the acute group

TABLE 2

Mean Values of Electrocardiograms of Experimental Chagas' Disease in Mice

Leads, complex, etc.		Normal	Acute	Subacute	Chronic
I	R-R′	.3012 mv.	.2236 mv.	.3468 mv.	.2804 mv.
I	S	.1215 mv.	.1327 mv.	.0961 mv.	.1826 mv.
II	P	.1857 mv.	.1611 mv.	.1586 mv.	.1939 mv.
II	R	.8957 mv.	.7491 mv.	.8622 mv.	.8891 mv.
II	S	.2754 mv.	.3100 mv.	.1664 mv.	.2396 mv.
II	R′	.3918 mv.	.3873 mv.	.3364 mv.	.4087 mv.
II	T	.1006 mv.	.1009 mv.	.0871 mv.	.1191 mv.
III	R	.7163 mv.	.5455 mv.	.7428 mv.	.6978 mv.
III	S	.3194 mv.	.3818 mv.	.3657 mv.	.2978 mv.
P-R		.0372 secs.	.0383 secs.	.0406 secs.*	.0399 secs.
QRS		.0239 secs.	.0304 secs.*	.0240 secs.	.0200 secs.*
Q-T		.0420 secs.	.0496 secs.*	.0414 secs.*	.0377 secs.
Erect †		68.75%	72.72%	71.43%	78.25%
Isoelectric†		21.87%	18.18%	21.42%	13.04%
Inverted†		9.37%	9.09%	7.14%	8.70%
Heart Rate		660.15	615.46	665.00*	635.65

* Statistically significant as compared with normal.
†Direction of QRS in Lead I.

was found to be significant suggesting myocarditis. There was also an increase of the S in all leads in the acute group. There was a noticeable change in the conformation of R′ and a lowering of the T-takeoff, again suggesting a conduction disturbance or myocarditis or both (Fig. VIII). None of the amplitudes showed any significant difference.

A significant difference of means was seen for the subacute group compared to normals for P-R interval, Q-T interval and heart rate, and for the chronic group compared to normals of QRS interval (Tables 1 and 2).

Histopathology and culture tests. Following the last ECG tracing and just before autopsy, the heart blood from all animals in-

TABLE 3

Statistical Analysis of Experimental Data From Table 5 ("Student's" t-test)*

X_1	X_2	N_1	N_2	$\overline{X_1}$	$\overline{X_2}$	t	d.f.	P	Significance
Normal II-R	Acute II-R	33	11	8.96	7.49	2.10	42	.04	+
Normal II-R	Subacute II-R	33	14	8.96	8.62	.54	45	.59	−
Normal II-R	Chronic II-R	33	23	8.96	8.89	.11	54	.91	−
Normal II-S	Acute II-S	30	9	3.03	3.79	−1.28	37	.20	−
Normal II-S	Subacute II-S	30	9	3.03	2.59	.81	37	.42	−
Normal II-S	Chronic II-S	30	17	3.03	3.24	− .37	45	.71	−
Normal II-R′	Acute II-R′	33	11	3.92	3.87	.16	42	.87	−
Normal II-R′	Subacute II-R′	33	14	3.92	3.36	1.94	45	.05	−
Normal II-R′	Chronic II-R′	33	23	3.92	4.09	− .62	54	.54	−
Normal P-R	Acute P-R	33	11	.037	.038	− .67	42	.50	−
Normal P-R	Subacute P-R	33	14	.037	.041	−3.20	45	.001	+
Normal P-R	Chronic P-R	33	23	.037	.040	−2.57	54	.01	+
Normal QRS	Acute QRS	33	11	.0239	.0304	−4.07	42	<.001	+
Normal QRS	Subacute QRS	33	14	.0239	.0240	− .08	45	.94	−
Normal QRS	Chronic QRS	33	23	.0239	.0200	3.85	54	<.001	+
Normal Q-T	Acute Q-T	33	11	.0420	.0496	−3.89	42	<.001	+
Normal Q-T	Subacute Q-T	33	14	.0420	.0414	3.84	45	<.001	+
Normal Q-T	Chronic Q-T	33	23	.0420	.0377	2.89	54	.004	+
Normal H.R.	Acute H.R.	33	11	660.15	615.46	2.22	42	.03	−
Normal H.R.	Subacute H.R.	33	14	660.15	665.00	−3.19	45	.001	+
Normal H.R.	Chronic H.R.	33	23	660.15	635.65	1.92	54	.05	−

* = Formula for t-test:

$$t = \frac{\overline{X}_1 - \overline{X}_2}{\sqrt{\Sigma X_1^2 - \frac{(\Sigma X_1)^2}{N_1} + \Sigma X_2^2 - \frac{(\Sigma X_2)^2}{N_2}}} \cdot \sqrt{\frac{N_1 N_2 (N_1 + N_2 - 2)}{N_1 + N_2}}$$

+ = Highly significant.

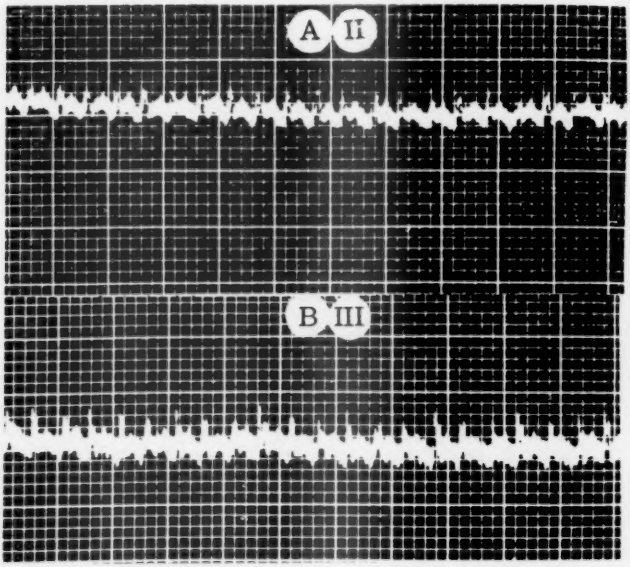

FIG. IV. Tracings of normal mice with modern high-speed two-channel hot-stylus electrocardiograph machine at a paper speed of 75 mm. per minute showing less detail in record than is possible with string galvanometer or oscilloscope. (A) Mouse No. 5. (B) Mouse No. 6.

oculated into suitable culture media was invariably positive for *Tr. cruzi*. Histopathology of the mice in the acute group revealed very numerous and widespread groups of leishmanial forms throughout the myocardium. Autopsies of mice in the chronic group did not demonstrate any abnormal scarring of myocardium, however, all mice showed myocarditis and some showed aflagellar forms in muscle and various organs. Although histopathological studies showed numerous and widespread groups of leishmanial forms of *Tr. cruzi;* they were not in sites closely related to conduction areas of the myocardium.

Discussion

It is well known that Chagas' disease as a rule is associated with focal myocarditis due to the invasion of the cardiac tissues by the aflagellar form of *Tr. cruzi*. In addition several investigators

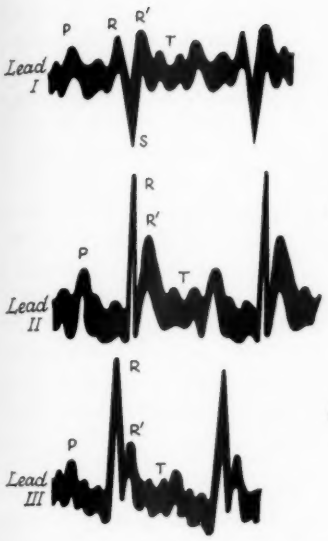

FIG. V. Enlargement of typical tracing from string ECG of healthy mouse with normal variations.

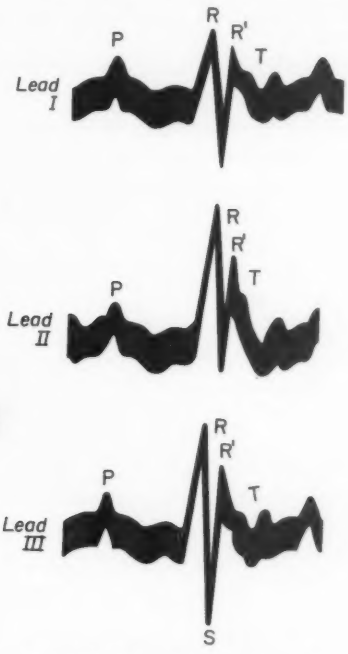

FIG. VI. A typical representative complex from normal mouse electrocardiogram demonstrating various components.

have shown that cardiac arrhythmias are strikingly frequent in man with chronic Chagas' disease. ECG abnormalities which have been reported in nearly 90 per cent of patients suffering with chronic Chagas' disease revealed the following; *i.e.*, right bundle branch block from 36.8 to 55.7 per cent, vent. extrasystoles 26.6 to 53.9 per cent, primary T-wave abnormalities 10.0 to 42.4 per cent, P-wave abnormalities 5.5 to 23.0 per cent, partial or complete A-V block 11.4 to 48.8 per cent, and, to a lesser degree, low voltage notching and slurring of QRS, auricular extrasystoles, auricular fibrillation, supraventricular tachycardia and ventricular tachycardia. Contrary to high incidence of RBBB, most authors report very low percentage of left bundle branch block (Chagas, 1930; de Barros, 1949; de Menezes, 1949; Dias,

Laranja, and Nobrega, 1945; Laranja, 1949; and Rosenbaum and Alvarez, 1955).

In this study, there was found no consistent ECG evidence of myocardial damage analogous to the conduction defects observed in the human cases of Chagas' disease. Although significant differences of the mean values were noted in the infected groups, these changes do not fall in the fixed patterns of change considered presumptive evidence of Chagas' disease in human beings. The significantly lengthened QRS segment and Q-T interval found in the acutely infected animals are however suggestive of myocarditis.

With the modified electrocardiograph and animal board used in this study easily readable electrocardiograms were secured from mice. It is noteworthy that Lombard (1952) by using a similar apparatus reported mean heart rate of mice (Mus musculus) as 376±49 beats/minute. It must be pointed out, however, that anesthetic premedication was used in Lombard's work. In the present study, a reduction in heart rate from mean value of unanesthetized mice also occurred when tracings were taken under anesthesia.

Summary

A modified string galvanometer electrocardiographic apparatus having a camera speed of 90 mm. per second, used in connection with new animal board, yielded easily readable electrocardiograms of normal mice and mice infected with *Trypanosoma cruzi*.

Eighty-nine electrocardiograms taken from 33 normal mice (*Mus musculus*) and 106 ECG tracings from 48 mice with acute, subacute and chronic Chagas' disease were examined and the calculated mean values for each group statistically analyzed. Significant differences of means between infected and uninfected animals were found in measurements of P-R, QRS and Q-T intervals and in heart rate.

Noticeable changes in conformation of curves in the acute group were as follows: increase in amplitude of S, doming and broadening of R′ and lowering of the T-takeoff.

Differences of the means between acutely infected and uninfected animals were indicative of definite myocardial damage suggestive of myocarditis; while the significant changes in the

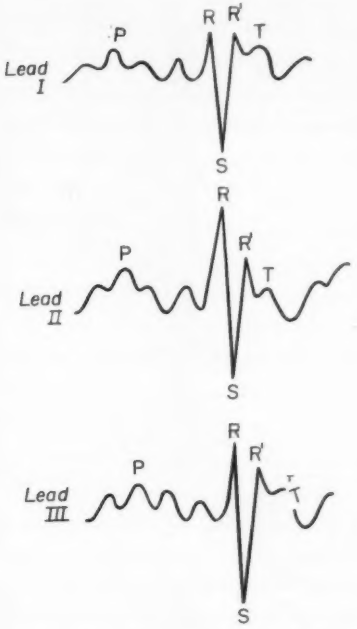

FIG. VII. Enlargement from photograph of oscilloscope tracing of normal mouse. (Compare with similar enlargement from string tracing for fidelity of string, Fig. III.)

subacute and chronic groups did not indicate a fixed pattern. In all cases, the significant differences proved to be in the time factors and not in amplitudes of the wave.

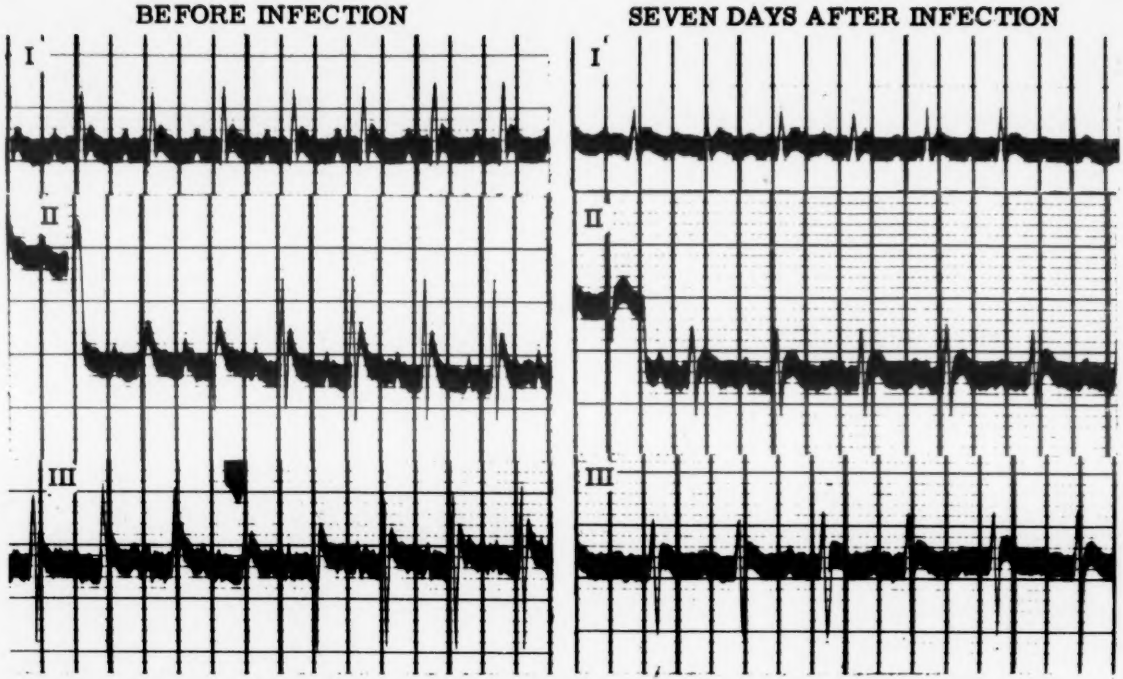

FIG. VIII. ECG's of mouse No. 29 before and after his infection with *Tr. cruzi*. Note changes after infection, *i.e.*, decreased rate, increased P-Q interval, decreased amplitude of all elements, definite Q_3, lowered T take-off and doming of T wave, biphasic P wave.

Fig. VIII. ECG of mouse No. 25 [illegible] rate, increased P-Q interval, decreased amplitude of all elements, definite Q_3, lowered T take-off and doming of T wave, biphasic P wave.

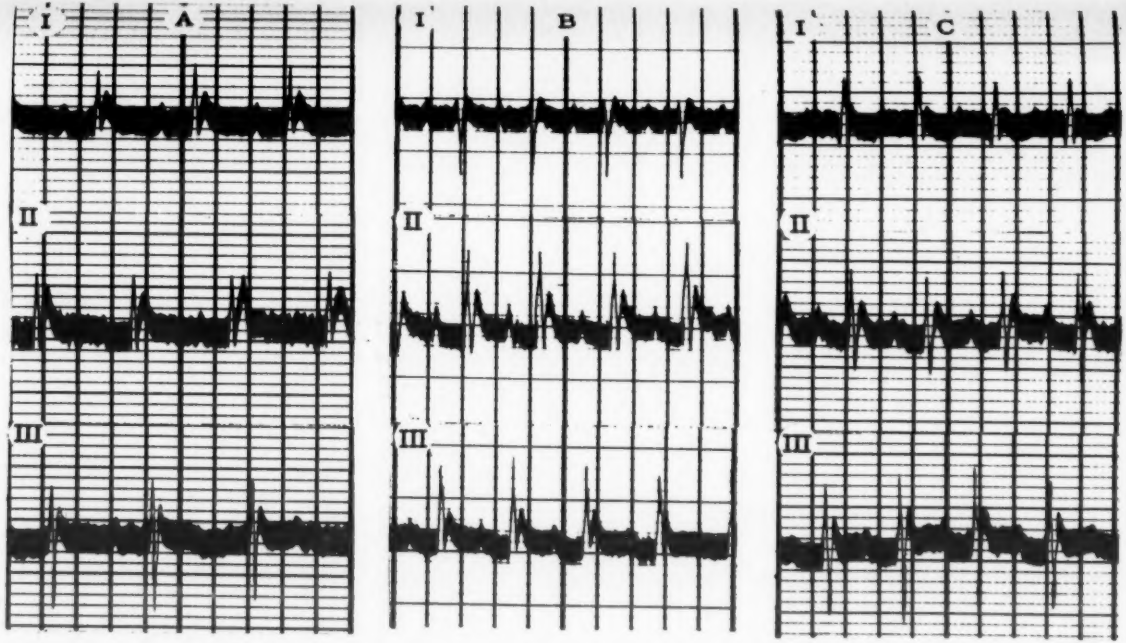

Fig. IX. Electrocardiograms of three mice (A, B, and C) after chronic course of Chagas' disease illustrating representative variations of the three bipolar leads. In the middle lead III shows slurred intrinsicoid deflection.

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A FURTHER USE OF ROSE'S TISSUE CULTURE CHAMBER

H. J. Woodliff[a]

The versatile multipurpose tissue culture chamber described by Rose (1954) is commonly used for glass substrate cultures where oil immersion phase contrast microscopy is required (Rose, 1954, Woodliff, 1958). Rose (1954) also mentions its use for plasma clot and cellophane substrate cultures and he has also adapted the chamber for use in compression, tension and dialysis experiments (Rose, 1957). This note describes the adaptation of the chamber for serum agar cultures.

The culturing of cells on serum agar has been developed by Pulvertaft (Pulvertaft and Jayne, 1953). In the original technique a small square of serum agar was placed on an ordinary 3″ × 1″ glass slide, the inoculum placed on top and the whole overlayed by a coverslip resting on four soft paraffin pillars. After sealing the paraffin the preparation was ready for culture and could be observed directly by phase contrast microscopy. Personal experience (Woodliff, 1956) in the application of this technique to blood and bone marrow cultures was disappointing. The cells survived for several days but did not mature or multiply. A modification of the technique (Pulvercraft, Haynes and Groves, 1956) using a special plastic culture slide in which fluid and gas phases can be equilibrated with the serum agar has given better results in terms of longer cell survival and in allowing the observation of mitosis in myelocytes (Woodliff, 1957). However in this slide the coverslip is sealed to the plastic chamber with paraffin wax which tends to loosen if attempts are made to perfuse the culture. The Rose chamber was therefore adapted for this type of culture. In place of the lower coverslip a 2″ square of thin "Perspex" brand polymethyl methacrylate with a ring cemented to the centre is used (Fig. I). A similar plate made of glass gives slightly better optical results, but is more expensive.

The plate after sterlizing by boiling in distilled water is placed in a sterile petri dish. A sterile gasket is placed on top and 2 or 3 drops of molten medium, composed of equal parts of agar (2 per cent in distilled water), serum and medium 199, is poured into

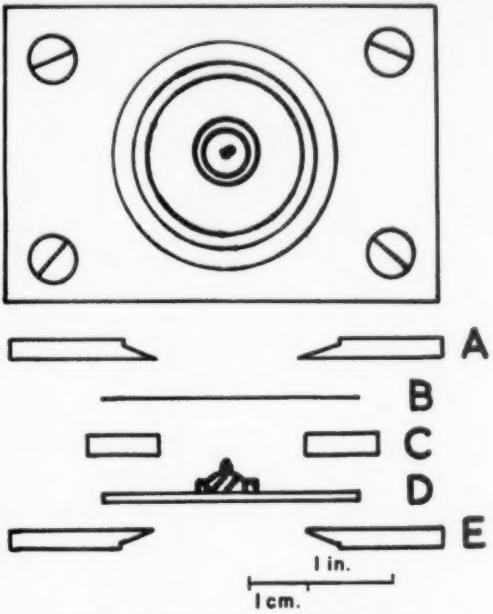

FIG. I. Diagram of chamber adapted for serum agar cultures. A. and E. stainless steel plates: B. coverslip: C. gasket: D. perspex or glass plate with the serum agar and inoculum.

the well of the ring. A 2″ square coverslip is then placed on top. When ready for use the coverslip is removed, the inoculum placed on the agar and the coverslip reapplied. The preparation is then sandwiched between the two stainless steel plates and after piercing the gasket with a needle the screws are tightened. Fluid and gas phases as desired can then be introduced by piercing the gasket with hypodermic needles. A simple device for perfusing the chamber has also been used (Fig. II). This allows alterations to be made in the fluid and gas phases of the medium surrounding the serum agar. Culture fluid from the reservoir is allowed to flow

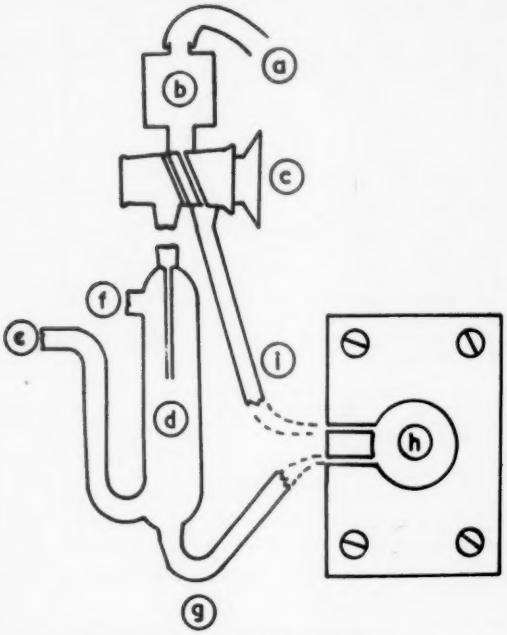

FIG. II. Diagrammatic representation of perfusion apparatus; Not to scale.
(a) polythene tubing; by applying a syringe to the end a negative pressure can be produced in the reservoir.
(b) reservoir.
(c) two-way tap.
(d) gas equilibration chamber.
(e) gas inlet; a filter is incorporated between the gas source and this inlet.
(f) gas outlet; a water seal, not illustrated, is attached here.
(g) tube from chamber to the reservoir; a needle through the chamber gasket is connected to the polythene tube by means of an adaptor.
(h) the culture chamber.
(i) tube to chamber.

into the gas equilibration chamber by gravity. After adjusting the two-way tap, negative pressure is applied to the top of the reservoir chamber by means of a syringe. This draws fluid from the gas equilibration chamber into the chamber. Using this technique the effect of different gas phases, drugs, etc. upon the living cells can be studied.

The type of observation which can be made using this chamber is illustrated in figure III.

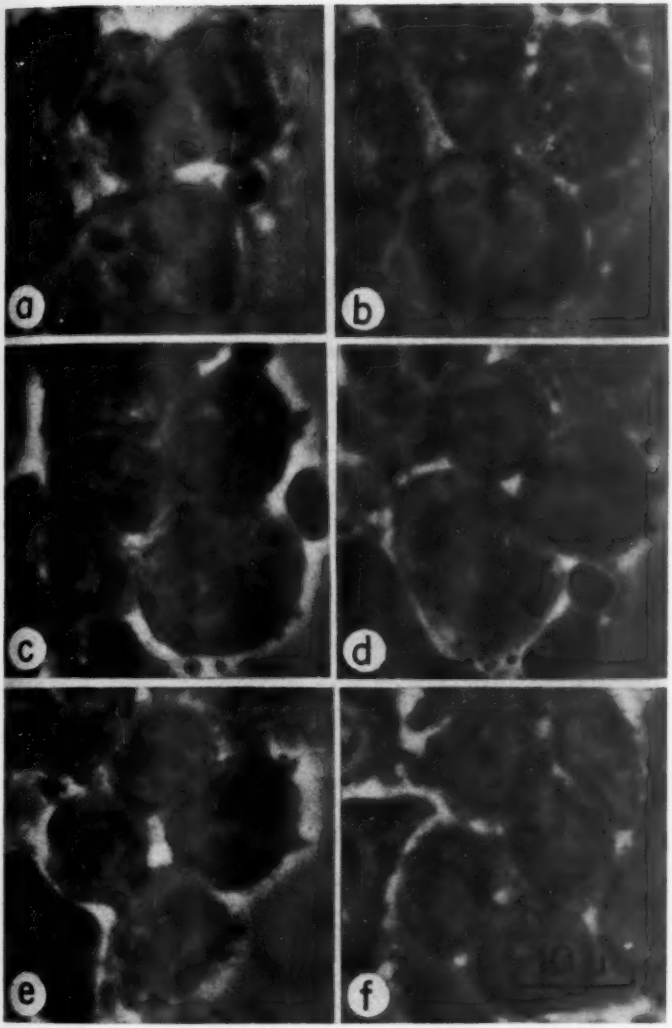

FIG. III. Photomicrographs illustrating observations made in the chamber. Stages of mitosis in cells from the marrow of a patient with acute leukemia.

(a) Four hour culture of two cells, one in telephase.
(b) The same field 13 minutes after (a); the nuclei of the daughter cells are reconstituting.
(c) The same field 53 minutes after (a); the other cell is now in mitosis (metaphase).
(d) The same field 75 minutes after (a); telephase.
(e) The same field 84 minutes after (a); commencing cytokinesis.
(f) The same field 120 minutes after (a); the four daughter cells.

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I wish to thank Mr. R. Flemans for help with the illustrations.

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CALLING ATTENTION TO

April, 1958

Clinical: W Dorfman moderates panel on obesity (*Internat Rec. Med* 171/1/58). IS Eskworth offers holistic approach to angina pectoris (*Am Heart J* 55/621/58). PH Forshan edits symposium on adrenal cortical therapy (*Metab* (7/3–89/58). E Goldberger hypothesizes pathogenesis of essential hypertension (*Am J Cardiol* 1/154/58). LG Goodwin & Co report on anthelmintic action of piperazine salts (*Brit Med J* 1/131, 136/Jan 18/58). AA Luisada & Co consider mechanism of heart sounds (*Am Heart J* 55/383/58). JR Mitchell opens talks on treating myocardial infarction (*Am J Cardiol* 1/224–283/58). JH Meyer & Co find meprobamate better than phenobarbital in psychoneurotic illness (*Geriatrics* 13/153/58). RW Rundles & SS Brewer note hematologic response in pernicious anemia to orotic acid (*Blood* 13/99/58). HSS Sarajas & Co describe stress in hyperthermia (*Nature* 181/612/Mch 1/58). H Schmidt Jr & Co find atropine depresses food & water intake (*Am J Physiol* 192/543/58). CL Steinberg & Co discuss water fluoridation & musculoskeletal diseases (*N Eng J Med* 258/322/Feb 13/58). T Tanabe & EJ Cafruny note adrenal enlargement on giving morphine repeatedly (*J Pharmacol* 122/148/58). DD Van Fossan & RT Clark Jr give post-mortem diagnosis of hypoxia by brain lactic acid concentration (*Am J Physiol* 192/577/58). BC Wexler & BF Miller note arteriosclerosis from corticotrophin (*Science* 127/590/Mch 14/58). A Wikler well reviews *Relation of Psychiatry to Pharmacology* with *full bibliography* (Wms & Wilkins, Balt, 58, 330 pp, $4.

Preclinical: BC Abbott, AV Hill, & JV Howarth report positive & negative heat production going with nerve impulse (*Proc Roy Soc B* 148/149/58). LG Abood & Co describe psychotogenic & anticholinergic action of N-methyl-3-piperidyl benzilates (*Proc Soc Exp Biol Med* 97/483/58). L Binet & Co show lipid metabolism effects in poisoning with *amanite phalloides* (*Compt rend Acad Sci* Jan 20/58, 339). GA Brecher & AT Kisson report ventricular diastolic suction at normal arterial pressures (*Circul Res* 6/100/58). VL Brooks & R Jastrow localize stretch reflexes by recurrent inhibition (*Science* 127/472 Feb. 28/58). H Brunner & Co study water diuresis (*Arch Exp Path Pharmacol* 233/1–67/58). A Carlsson & Co note 3-hydroxy-tyramine (precursor of noradrenalin) in brain (*Science* 127/471/Feb 28/58). PB Dews analyses stimulant action of methamphetamine (*J Pharmacol* 122/137/58). S Garattini & Z Ghetti edit *Psychotropic Drugs* (D Van Nostrand, Princeton 57, 621 pp, $20). GF Gause describes search for anticancer antibodies (*Science* 127/506, Mch 7/58). WE Huckabee studies pyruvate & lactate relations in anaerobic metabolism (*J Clin Metab* 37/244, 264/58). H Kless reports inhibition of adreno-cortical activity with 1,2-bis-p-amino-phenyl-2-methyl-propanone-1 (*Arznei Forsch* 8/83/58). SB Koritz & FG Peron study mechanism of ACTH action (*J Biol Chem* 230/343/58). Ch S Koshtoyants offers *Comparative Physiology of the Nervous System* (*USSR Acad Med Sci*, Moscow, 57, 635pp, 36r). NI Krinsky & Co study carotenoid transport in

human plasma (*Arch Biochem Biophys* 73/233/58). CC Little reviews biological aspects of cancer research (*J Nat Cancer Inst* 20/441/58). RA MacDonald & Co find dermal fibrosis after subcut injections of serotonin (*Proc Soc Exp Biol Med* 97/334/58). Our B Marks & Co find reserpine causes loss of granules from enterochromaffin cells after serotonin (*Proc Soc Exp Biol Med* 97/413/58). FC McLean gives ultrastructure & function of bone (*Science* 127/451/Feb 28/58). MJ Osborn & Co find aminopterin inhibits dihydrofolic reductase (*Proc Soc Exp Biol Med* 97/429/58). E Ronwin discusses properties of thrombinogen (*Canad J Biochem Physiol* 36/75/58). RL Shull & RB Alfin-Slater find cholesterol increase in muscles of dystrophic mice (*Proc Soc Exp Biol Med* 97/403/58). L Stein notes secondary reinforcement of conditional reflexes on subcortical stimulation (*Science* 127/466/Feb 28/58). JM Stowers & Co review action of sulfonylureas in humans (*Lancet* 1/278/Feb 8/58). SF Takagi & Y Oomura note nicotine action on CNS synapses (*Am J Physiol* 192/447/58). B Uvnas reviews mechanism of histamine release (*J Pharm Pharmacol* 10/1/58). On Yang & Ti Lee review symmetry principles (*Science* 127/565, 569, Mch 14/58).

General: WR Amberson considers fashions in trends of study of electricity (*Am Sci* 46/33/58). HL Blumgart introduces Festchrift to WB Castle (*Arch Inter Med* 101/173–513/58). R Calfer gives journalistic heave-ho at *Medicine & Man: The Story of the Art & Science of Healing* (Mentor Books, NY, 58, 256pp, 50c). FE Egler notes abuse of roadway lands (*Science* 127/573/Mch 14/58). CC Gillispie looks at Greek science (*Am Sci* 46/62/58). A Goldstein well shows no value of rank grades in medical training (*J Med Educ* 33/193/58). WB Looney recommends more caution on radiation exposure (*Science* 127/630/Mch 21/58). MFA Montagu wraps up *The Reproductive Development of the Female* (Julian Press, NY, 58, 252pp, $5). HJ Muller discusses human values in evolution (*Science* 127/625/Mch 21/58). EL Sevringhaus notes interdependence of medicine and pharmacy (*World Med J* 5/111/58).

MAY, 1958

General: AAAS issues report of 1958 Parliament of Science held to develop opinion on public policy matters involving science (*Science* 127/852/Apr 18/58). W Bergman & Co discuss centennial significance of cellular pathology inaugurated by R Virchow (1821–1902) (*Deutsch Med Wchnschr* 83/361–377/Mch 7/58). S Dahl revels in the *History of the Book* (Scarecrow Press, NY, 58, 280pp, $6). DL Drabkin eulogizes *Thudicum: Chemist of the Brain* (Univ Pennsylvania Press, Phila. 58, 309pp, $5) referring to JLW Thudicum 1829–1901 whose classic was *Die Chemische Konstitution des Gehirns* (F. Pietzcher, Tubingen, 1901, 339pp). JC Greene notes low appreciation of science in age of Jefferson (*Isis* 49/13/58). AB Hollingshead & FC Redlich analyse *Social Class & Mental Illness* (J Wiley, NY, 58, 442pp, $8). DJ Ingle gives *Principles of Research in Biology & Medicine* (Lippincott, Phila, 58, 123pp). A Macleish startles with Job to date in *JB: A Play in Verse* (Houghton-Mifflin, Boston, 58, 153pp, $3.5). AJ McConnell describes Wm Rowan Hamilton (1805–1865) & his genius on principle of least time in

optics & least action in dynamics (*Adv Sci* 14/323/58). GF Otto reflects on ecology of parasitism (J Parasit 44/1/58). GC Robinson enjoys *Adventures in Medical Education* (Harvard Univ. Press, Cambridge, 58, 212pp, $5). A Romer gives history of transformation theory of radioactivity established in 1902 by E Rutherford & F Soddy in Montreal (*Isis* 49/3/58). E Teller & AL Latter are aggressively selective in *Our Nuclear Future: Facts, Dangers & Opportunities;* why? (Criterion Books, NY, 58, 184pp, $3.5). RJ Williams opens a door to chemical anthropology (*Amer Sci* 46/1/58). CH Waddington gives *The Strategy of Genes* (Macmillan, NY, 58, 167pp, $4).

Clinical: DW Allen & Co discuss defense reactions to anxiety among physicians as factors in resistance to learning (*J Med Educ* 33/373/58). S Banerjee & PS Agarwal note increase in 5-hydroxyindoleacetic acid excretion in schizophrenics (*Proc Soc Exp Biol Med* 97/657/58). RO Bauer & AE Miller cat (call attention to) medical problems of jet age (*Bull Millard Fillmore Hosp*, Buffalo, 5/16/58). K Bucher analyses patho-physiology & pharmacology of cough (*Pharmacol Rev* 10/43/58). SRM Bushby reviews chemotherapy of leprosy (*Ibid* p 1). WS Howland describes cardiovascular & clotting disturbances during massive blood replacement (*Anesth* 19/140/58). GF Lambert & Co induce atherosclerosis on high fat diet devoid of cholesterol (*Proc Soc Exp Biol Med* 97/544/58). HI Lief edits symposium on psychosomatic aspects of digestive disease (*Am J Digest Dis* 3/247–337/58). JG Millichap suggests high carbonic anhydrase activity needed for grand mal seizure (*Proc Soc Exp Biol Med* 97/606/58). AJ Quick analyses *Hemorrhagic Diseases* (Lea & Febiger, Phila, 58, 451pp, $10). JW Sevringhaus & SC Cullen note depression of myocardium & body oxygen with Fluothane, $CF_3CHClBr$ (*Anesth* 19/165/58). A Sjoerdsma & Co find 3-methoxy-norepinephrine in pheochromocytoma (*Science* 127/876/Apr 18/58). CS Welch & SR Powers reveal *The Essence of Surgery* (WB Saunders, Phila, 58, 320pp, $7).

Preclinical: HA Abramson & Co study lysergic acid diethylamide antagonists (*Arch Neurol Psychiat* 79/201/58). K Bowden & GA Mogey give story of muscarine (*Pharm Pharmacol* 10/145/58). KM Bykov describes relations of *Cerebral Cortex & Internal Organs*, trans by WH Gantt (Chem Publ Co, NY, 57, 448pp, $15). A Engstrom & JB Finean describe *Biological Ultrastructure* (Academic Press, NY, 58, 326pp, $8). CH Graham & Y Hsia discuss color visual defects & color theory (*Science* 127/675/Mch 28/58). LV Heilbrunn discusses *The Viscosity of Protoplasm* (Bd II *Protoplasmatologia*, Lange & Springer, Berlin, 58, 109pp, 1957, $7). BH Hoyer & Co discuss anion exchange purification of viruses & ricketsiae (*Science* 127/859/Apr 18/58). RJA Kirkland depicts plant & animal cells as complicated mosaics of structural units with high functional organization (*Adv Sci* 14/353/58). M Lee & Co well edit overwhelming 2109 abstracts for Phila sessions of Federated Societies for Experimental Biology (*Fed Proc* 17/1–576/58). TS Naumova indicates character of interrelations between cerebral hemispheres depends on levels of their excitation (*Sechenov Physiol J USSR* 43/288/58). RH Robertson & PJ Schaible find zinc necessary for growth & food utilization, in chicks (*Science* 127/875/Apr 18/58). AM Shanes surveys electrochemical

aspects of physiological & pharmacological action in excitable cells (*Pharmacol Rev* 10/59/58). E Schutz surveys *Physiologie des Berzens* (Lange & Springer, Berlin, 58, 570pp, DM 88). S Spector & Co monoamine oxidase inhibitors (Iproniazid & hydrazine cmpds) increase brain serotonin & norepinephrine & excitation (*Science* 127/704/Mch 28/58). H Spencer & Co show radiostrontium excretion increases after stopping ethylene-diamine-tetra-acetic acid (*Proc Soc Exp Biol Med* 97/565/58). HG Swann & Co show relation of lymph to kidney distending fluids (*ibid* p 517). G Ungar & DV Romano find SH increase in brain on afferent stimulation (*ibid* p 324). TP Waalkes & Co find high serotonin & norepinephrine in bananas (*Science* 127/648/Mch 21/58).

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Evolution: DA Biriukov outlines evolution of knowledge on excitation (*Sechenov Physiol J USSR* 43/447/58). T Dobzhansky reflects on the Darwin-Wallace ideas (*Science* 127/1091/May 9/58). Sir Gavin de Beer comments on the Darwin-Wallace centenary (*Endeavor* 17/61/58).
Central Nervous System: FM Forster introduces colloquium on neurochemistry (*Neurol Suppl* 1, 8/1–132/58). PH Hoch & J Zubin delve into *Psychopathology of Communication* (Grune & Stratton, NY, 58, 318pp, $7). JR Hughes edits WR Hess's *Functional Organization of the Diencephalon* (Grune & Stratton, NY, 58, 192pp, $7). PA Jewell & EB Verney try to locate neurophypophysial osmoreceptors (*Phil Trans Roy Soc London B* 240/672/58). KF Killam & Co show barbiturate potentiation by uncoupling oxidative phosphorylation with dinitrophenol (*Proc Soc Exp Biol Med* 97/744/58). A Koch & DM Woodbury show carbonic anhydrase inhibition alters brain excitability (*J Pharmacol Exp Therap* 122/335/58). JCLilly describes brain electrode implantation (*Science* 127/1181/May 16/58). HW Magoun reveals *The Waking Brain* (CC Thomas, Springfield, Ill, 58, 148pp, $5). AJ Mandell & LMN Bach show medullary reticular formation not only participates in respiratory, vasomotor & spinal reflexes, but also in conscious behavior involving emotional signs (*Proc Soc Exp Biol Med* 97/880/58) J & ME Olds find positive reinforcement by stimulating hypothalamus with iproniazid (*Science* 127/1175/May 16/58). JL O'Leary & LA Cobon review new concept of *reticular core* in CNS function (*Physiol Rev* 38/243/58). HH Pennes edits *Psychopharmacology* (PB Hoeber, NY, 58, 378pp, $8). DP Purpura & Co report central synaptic effects of guanidino & amino acid derivatives (*Science* 127/1179/May 16/58). E Roberts & Co trace brain metabolism of gamma amino butyric acid (*Proc Soc Exp Biol Med* 97/796/58). WF Windle discusses *The Biology of Neuroglia* (CC Thomas, Springfield, Ill, 58, 336pp, $8.5).
Clinical: L Alexander notes *Objective Approaches to Treatment in Psychiatry* (CC Thomas, Springfield, Ill, 58, 139pp, $3). J Gillies opens symposium on anesthesia (*Brit Med Bull* 14/1–70/58). M Ghione finds 4-chlorotestosterone protects against infections & promotes healing (*Proc Soc Exp Biol Med* 97/773/58). IL Uanis considers *Psychological* Stress: Psychoanalytical & Behavorial Studies of Surgical Patients (Wiley, NY, 58, 480pp, $7.5). E Jokl

gives *Clinical Physiology of Physical Fitness & Rehabilitation* (CC Thomas, Springfield, Ill, 58, 208pp, $8.5). JD Keith & Co cover *Heart Disease in Infancy & Childhood* (Macmillan, NY, 58, 832pp, $22.5). H Sobel & S Graboyes show graded cardiac hypertrophy to reduced barometric pressure & high temperature (*Proc Soc Exp Biol Med* 93/725/58).

Preclinical: WA Adams describes *Comparatice Morphology of the Carotid Body & Carotid Sinus* (CC Thomas, Springfield, Ill, 58, 268pp, $9). SA Ajl analyses terminal respiration in bacteria (*Physiol Rev* 38/196/58). DI Arnon & Co discuss assimilatory power in photosynthesis (*Science* 127/1026/May 2/58). RP Cook edits *Cholesterol: Biochemistry & Pathology* (Academic Press, NY, 58, 530pp, $15). G Corte & W Johnson indicate N-acetyl-p-aminophenol inhibits steroid glucuronide formation in liver (*Proc Soc Exp Biol Med* 97/751/58). JT Edsall arranges *Conference On Hemoglobin* (National Research Council, Washington DC, 58, 304pp, $3). AB Eisenstein & AS Wenneker note reduction in adrenocorticosteroid excretion in pantothenic acid deficiencies (*Proc Soc Exp Biol Med* 97/844/58). SD Feurt & Co note species variation in pharmacology & toxicology of nicotine (*Science* 127/1054/May 2/58). ME Langhorn reviews aqueous humor & control of intra-ocular pressure (*Physiol Rev* 38/215/58). M Lashowski & Co find trypsin inhibitor (in colostrum) facilitates intestinal absorption of such protein as insulin (*Science* 127/1115/May 9/58). IH Page brings information on 5-hydroxytryptamine to date (*Physiol Rev* 38/277/58). ZT Samilova finds vagal effects on coronary vessels (*Sechenov Physiol J USSR* 43/394/57). B Schmidt-Nielsen surveys urea excretion in mammals (*Physiol Rev* 38/139/58). NA Thorn discusses mammalian antidiruetic hormone (*Ibid* p169). S Udenfried & H Weissbach show rapid serotonin turn-over in brain (*Proc Soc Exp Biol Med* 97/748/58). VM Vinogradow reports CHS effect of "Pascain,"—diethyl amino-ethanol ester of p-amino-salicylic acid (*Sechenov Physiol J USSR* 43/530/57). S Waksman monographs *Neomycin* (Wms & Wilkins, Baltimore, 58, 417pp, 58).

General: WR Amberson illustrates influence of fashion in developing knowledge of electricity & magnetism (*Amer Sci* 46/33/58). N Bohr philosophizes in *Atomic Physics & Human Knowledge* (Wiley, NY, 58, 101pp, $4). B Commoner talks sense on radiation fallout problem (*Science* 127/1023/May 2/58). GW Corner opens his life & letters in *Anatomist at Large* (Basic Books, NY, 58, 233pp, $4). NG Hawkins offers *Medical Sociology* (CC Thomas, Springfield, Ill, 58, 290pp, $7).

C. D. Leake

VIRAL INHIBITORS IN NORMAL ANIMAL SERA[a]

RAE ALLEN,[b] RICHARD A. FINKELSTEIN,[b,c] AND S. EDWARD SULKIN[b]

For many years investigators have been aware of the presence of viral inhibitors in normal animal sera. Only a limited number of reports have been devoted solely to investigation of the nature of such inhibitors but references to unexplained viral inactivation by serum are scattered throughout the literature. Naturally occurring inhibitors of viral activity, apart from specific antibody, may be classified empirically into three main categories by virtue of their chemical properties and reaction to heat: (1) inhibitors of lipid nature extractable by fat solvents; (2) proteins, protein complexes, and mucopolysaccharides which are relatively stable to heat; (3) heat labile proteins and protein complexes. Investigation into the nature of these inhibitors and their biological activity may be complicated by the simultaneous occurrence of two or more such inhibitors, possibly in addition to specific antibody, in the same serum. Furthermore, there is considerable quantitative and qualitative variation in serum content of these substances between species, individuals in a given species, and even in serum from the same individual at different times.

By virtue of their ability to modify some of the observable properties of viruses, such as hemagglutinating capacity and infectivity, they may be confused by the unwary virologist and serologist with specific antibody. Since normal animal serum is widely used as a nutrient for cells in tissue culture for the isolation of viruses, the presence of nonspecific viral inhibitors may reduce the sensitivity of virus isolation procedures. Thus, whenever serum is used in the isolation and identification of viruses, the investigator must be aware of the possible sources of error introduced by nonspecific inhibitors and must control or eliminate their effect.

It should be recognized that these serum components may provide more than a laboratory nuisance. They constitute a potential tool for learning more about the properties and nature of viruses.Thus the investigator may exploit the different susceptibilities of various viruses in the classification of these agents and the study of their biological and chemical activities. Sensitivity to nonspecific inhib-

tors in serum may be correlated with other known properties of viruses (ether sensitivity, heat stability, size, and reaction to other physical and chemical agents) to achieve either of the above goals.

The widely used term "nonspecific inhibitor" is, in this regard, a misnomer. These compounds certainly must have specific substrates and specific modes of action. Unfortunately, these properties are only poorly understood at present. Therefore, the reader should be aware that in its present usage the term "nonspecific" implies only the absence of a known specific antigenic stimulus.

In addition, these substances may play important roles in natural host resistance. It is not unreasonable to assume that serum components which inactivate viruses *in vitro* may do so *in vivo* for the benefit of the host although conclusive evidence is lacking. The recent discovery of the properdin system by the late Dr. Louis Pillemer constitutes an important break-through in the study of natural immunity.

This study is concerned with the nature and properties of virus inhibitors which occur naturally in animal sera. Their significance in the study of viruses and the diseases caused by them will be considered.

Heat Stable Inhibitors

Flexner and Lewis (1910) were among the first to report the presence of nonspecific viral inhibitors in animal sera. While attempting to produce a therapeutic immune serum against poliomyelitis, they found that normal sheep serum possessed a slight neutralizing activity for poliovirus. This observation was confirmed by Stewart and Haselbauer (1928). The reason for this chance neutralization was obscure to these workers. However, they emphasized that it should not be confused with the consistent neutralizing activity of both human and monkey convalescent sera.

Jungeblut and Engle (1932a) reported on the capacity of certain normal animal sera to neutralize the virus of poliomyelitis. Although sera of immature rhesus monkeys were inactive, sera from four of nine adult monkeys neutralized poliovirus. The four active sera were from adult females bled ten days after menstruation. It is of interest to note that two of these bled a few days before onset of their next menstrual cycle were inactive indicating the transitory nature of the inhibitor. Sera from three of four *Cebus* monkeys neutralized poliovirus. This finding is of interest since *Cebus* monkeys

are insusceptible to poliomyelitis. Sera from four sheep and one cock failed to inactivate virus. The authors concluded that certain normal animal sera possess neturalizing substances for poliovirus which behave identically with antibodies but may occur under conditions which preclude previous contact with specific antigen. Later, virucidal substances were demonstrated in sera of normal young chimpanzees (Jungelut and Engle, 1934).

At this point in poliomyelitis research the monkey was the only host animal available to laboratory workers seeking to learn more about the etiology, epidemiology, control and treatment of the disease. The neutralization test in monkeys was the only means of identifying newly isolated strains of virus or of carrying out serologic surveys. Because monkeys were essential to the test, most reports were based on small numbers of animals and errors were inevitable. With the adaptation of a strain of poliovirus to the mouse (Armstrong, 1939) larger numbers of animals could be used, thus increasing the significance of the results. Peculiar immunological discrepancies still occurred. One possible explanation for such inconsistencies was the accumulating evidence for the existence of immunologic differences among virus strains (Aycock, 1942). At that time the neutralization of poliovirus by certain normal human sera was attributed to "physiological maturation" (Jungeblut and Engle, 1932b) though it is now recognized that this is an expression of subclinical infection. Another explanation lay in the fact that certain sera from man and animals contained nonspecific inhibitors, the origin and nature of which remained a mystery to the investigator. Sera from over 300 vertebrates other than man were tested for Type 2 (Lansing) poliovirus antibodies by the mouse neutralization test (Hammon and his colleagues, 1947). A comparison was made of sera from domestic cows, horses, chickens and birds and similar animals held on desert ranges or in isolation. Many sera from domestic animals were found capable of neutralizing poliovirus while few were positive among the isolated groups or wild species. The finding of poliovirus neutralizing substances in the sera of a large number of domestic animals suggested the possibility of extrahuman reservoirs of poliomyelitis virus. Gordon (1945) reported the neutralization of poliovirus by dog serum. Three of thirty-seven canine sera collected following the 1943 epidemic of poliomyelitis in Chicago were found to neutralize the Lansing mouse-adapted poliovirus. Hammon and his associates (1947), however, suggested that

the specificity of the neutralizing substances found in the sera of domestic animals could not be established. There was no adequate basis for assuming that anti-Lansing virus substances in the sera of certain domestic mammals resulted from infection with poliovirus. Such virus neutralizing substances might have been the result of infection with an agent antigenically related to poliovirus.

In 1951, Chang and Wenner described reciprocal neutralization between monkey immune sera to an isolate from bovine encephalomyelitis and poliovirus Type 2 (Lansing). These findings, they stated, would explain the heretofore perplexing observations regarding the occurrence of neutralizing antibodies for Lansing poliovirus in body fluids of cattle and other mammals, and domestic birds (Hammon *et al.*, 1947). To determine a possible etiologic relationship of Type 2 poliovirus to bovine encephalitis further experimental studies were performed (Wenner *et al.*, 1953, 1954). Inoculation of calves with Type 2 poliovirus failed to cause overt or occult infection nor was infection induced by the virus strains previously isolated. These agents even failed to provoke in calves a specific antibody response. It therefore seemed unlikely that the occurrence of substances neutralizing the Type 2 poliovirus in serum from cattle (Hammon *et al.*, 1947) was the result of poliomyelitis infection. It therefore appears likely that the cross reactivity observed in their initial studies might have been caused by "nonspecific inhibitors."

During the course of studies on the antipoliomyelitic properties of milk from different animal species, Sabin and Fieldsteel (1953) found that the colostrum from each of 16 cows tested neutralized Type 2 (Lansing) poliovirus. The undiluted sera of 16 of 17 cows neutralized the Lansing virus; indices ranged from 50 to 500. When sera from calves, aged 5 to 12 months, were tested a much smaller percentage of positives was obtained. Neutralizing substances for Types 1 and 3 polioviruses were also demonstrated in bovine sera. The antipoliomyelitic activity of bovine serum was found to resemble the activity of antibody in human serum: incubation of the mixtures was not necessary to demonstrate neutralization and heating at 60°C for 30 minutes did not remove the activity which was associated with the globulin fraction of the serum. Attempts were made to infect calves with a virulent strain of poliovirus at a time when their serum was devoid of neutralizing activity against the virus. In accord with the work of Wenner *et al.*, (1954) it was im-

possible to produce even inapparent infection in the calves. From the results of these studies the authors proposed that cattle might be infected with an agent or agents antigenically related to the polioviruses and that it might be worthwhile to search for such an antigenic relative in cattle.

Bartell and Klein (1955) also observed inhibitory factors in the sera of cattle which neutralized all three types of poliovirus and believed these neutralizing substances to be true antibodies. Their results showed that some cows had no antibody and others had antibody to Types 1, 2, and 3 in various combinations. They reasoned that this high degree of specificity was incompatible with the effects of any nonspecific neutralizing substances. Neutralizing activity was not detected in serum of young animals, increased with age and was not present in all adults of the species. The neutralizing substance had the heat stability characteristic of antibody. The authors concluded that these substances in bovine sera were antibodies specific for poliovirus. Numerous attempts were made to isolate from cattle viruses that would be neutralized by Types 1, 2, or 3 poliovirus sera but the antigenic stimulus eliciting the antibodies was not found. Although the evidence (Hammon *et al.*, 1947; Sabin and Fieldsteel, 1953; Bartell and Klein, 1955) suggests the identity of spontaneously occurring neutralizing substances with specific antibody for polioviruses, it cannot be regarded as conclusive unless a specific antigenic stimulus can be demonstrated.

Recently, Takemori and co-workers (1957, 1958) isolated poliovirus mutants resistant to the effect of inhibitory normal bovine serum (INBS) in a tissue culture system. Inhibitor resistant strains could be developed by serial passage of the initially sensitive population in the presence of INBS or could be selected directly by plaque morphology characteristics on plates with an agar overlay containing INBS. Despite the ease with which INBS resistant mutants were obtained, the authors were unable to isolate poliovirus particles genetically resistant to homologous antibody. Experimental evidence was obtained for the existence of several qualitatively distinct inhibitors. This survey would seem to indicate that poliovirus neutralizing substances in normal bovine serum are not antibodies but nonspecific serum inhibitors.

In one of the early studies on the cultivation of poliovirus in tissue culture it was observed (Robbins *et al.*, 1952) that 20 percent

inactivated horse serum in the tissue culture medium, while enhancing the growth and survival of human uterine tissue, exerted an unfavorable influence upon the multiplication of poliovirus. It was found, however, that viral activity was not affected by a concentration of 5 percent horse serum. Consequently, 20 percent horse serum was used only during the initial stage of cell cultivation and when virus was inoculated the concentration of serum was reduced to 5 percent. This inhibition of infectivity by relatively high concentrations of inactivated normal horse serum was also demonstrable in the mouse neutralization test.

The literature cited tnus far has dealt with the effect of heat stable normal serum inhibitors on the polioviruses in neutralization studies and in cultivation of the viruses in tissue culture systems. The inhibition of other viral agents by heat stable substances in normal animal sera has also been reported. Svedmyr *et al.* (1956), in a study of the poliomyelitis epidemic which occurred in Stockholm in 1953, reported the isolation of a number of viral agents which were not neutralized by antibodies against the three types of poliovirus. The authors stated that some difficulty was encountered when these "orphan" strains were tested in tissue culture against poliovirus antisera as well as sera from nonimmunized rabbits and guinea pigs. These sera, when used in high concentration, tended to inhibit the cytopathologic response of the tissue culture cells to the "orphan" viruses. The effect was much less marked when sera were diluted 1:5 or more. These "orphan" viruses probably belong in the ECHO virus group of Enteroviruses (Committee on the Enteroviruses, 1957).

Kjellen (1957), during studies on *in vitro* neutralization of adenoviruses, found that some normal rabbit sera influenced the virus dose-cell culture response relationship. With mixtures of adenovirus plus inactivated normal rabbit serum there was a delay in appearance of cytopathogenic changes similar to that obtained with mixtures of virus and low concentrations of hyperimmune sera. Gold and Ginsberg (1957) also reported the inhibition of adenoviruses by normal animal sera. During investigations to obtain a satisfactory nutrient medium for propagation of adenovirus in HeLa cells, they obsreved that ox, calf and horse serum markedly inhibited the viral cytopathogenic effect. The heat stable inhibitor formed a nondissociable complex with strains of Types 1–7 adenovirus and was not destroyed by RDE, trypsin, or incubation with infectious virus.

Recent adenovirus isolates were inhibited to the same extent as older laboratory strains. The inhibitor was precipitated by ammonium sulfate with the globulin fraction of the serum. Although the nature of the virus inhibitor was not further defined, the authors emphasized the importance of the composition of fluids employed for the propagation of adenoviruses in cell culture.

A recent report by Chang and Geyer (1957) is of interest in this regard. In a human cell culture system, these authors found that horse serum albumin could substitute for whole horse serum in the medium. In comparative virus titrations, Type 1 poliovirus, Type 3 adenovirus and Type 1 ECHO virus multiplied to significantly higher titers in albumin medium than in medium containing whole horse serum. Coxsackie virus (B1) grew equally well in both media. These results suggest that the albumin medium may be more suitable than the whole serum medium in certain primary virus isolation procedures using human cells.

With respect to yellow fever virus, Strode (1951) stated that one of the most important sources of variability in mouse protection tests in yellow fever depends on the inherent properties of the serum of different animal species. The presence of nonspecific neutralizing substances for yellow fever virus in animal sera was first reported in a survey of yellow fever immune bodies in the blood of African animals (Findlay *et al.*, 1936). Sera from African primates, captured in areas where yellow fever is endemic, neutralized the virus. Surprisingly, the serum of one of six English sheep used as controls protected mice against yellow fever virus although only in low dilution. Since this sheep could never have been exposed to yellow fever, the authors concluded that false positive protection tests may occasionally be given by the serum of certain *Ovidae*. In subsequent reports (MacCallum and Findlay, 1937; Findlay, 1941) dealing with immunological studies on yellow fever in Africa, the blood of many domestic animals (cows, sheep, horses, pigs, camels, and dogs) from endemic areas as well as from areas in which yellow fever in man is unknown were tested. In Kenya, where human cases have never occurred, 11 of 82 cows tested gave positive results in neutralization tests and 3 of 70 sheep examined also had protective substances in their blood. Bugher (1940) demonstrated inhibiting substances in the blood of the domestic pig and in sera of certain birds captured in an area in which yellow fever had not occurred for three years. These reactions were weak suggesting that they might

be artifacts characteristic of the test species. Bugher *et al.* (1944) in a later study of the epidemiology of jungle fever in eastern Colombia reported that the sera of many animal species possessed nonspecific virucidal characteristics and that it was necessary to establish a normal base line for each species concerned. Koprowski (1946) found that the sera of certain species of marsupials and rodents captured in Brazil neutralized yellow fever virus. The neutralization property was found to disappear and reappear on occasion. These same sera also neutralized Japanese, St. Louis, and West Nile encephalitis viruses which are not known to occur in Brazil. This fact, together with the transitory nature of the neutralizing substance, provided the basis for concluding that the reactions were nonspecific. In view of the reports cited above, the epidemiological significance of positive protection test results obtained in large scale surveys of wild and domestic animals in yellow fever endemic areas is questionable.

The discovery that influenza viruses agglutinate red blood cells (Hirst, 1941; McClelland and Hare, 1941) provided a convenient method for the study of virus-host relationships. This simple method, however, was soon complicated by the demonstration of heat stable nonspecific inhibitors of viral hemagglutination in normal animal and human sera. The presence of these inhibitors in ferret, guinea pig, fowl, and human sera was noted by many investigators (Hurst, 1942, 1948; Burnet and McCrea, 1946; McCrea, 1946; Anderson *et al.*, 1946; Friedwald *et al.*, 1947; Smith and Westwood, 1948). Following the demonstration by Francis (1947) of an hemagglutinin inhibitor which was more active against heated virus than live virus, this type of inhibitor has become known as the "Francis or α inhibitor" and the increased sensitivity of heated virus is known as the "Francis effect". Many studies have been directed toward characterization and understanding of the nature and mode of action of this inhibitor or group of inhibitors of viral hemagglutination. "Francis inhibitor" is present in normal tissues, secretions, and sera of man and animals (Friedwald, *et al.*, 1947) and is believed to be a mucoprotein (McCrea, 1948). The following characteristics are cited by Chu (1951): heat stable, destroyed by receptor-destroying enzyme (RDE) and periodate, inhibits heated virus to much higher titer than live virus, active against both mouse-adapted and unadapted strains, forms transient combination with active virus and is subsequently destroyed by it, does not as a rule

neutralize influenza virus *in vivo*. The Francis inhibitor is believed to be structurally analogous to the red cell and tissue receptors for influenza virus.

A second type of inhibitor of influenza virus hemagglutination has been describer by Smith *et al* (1951). This second factor, designated β inhibitor, is not destroyed by RDE, is not inactivated by heating at 56°C for 30 minutes following RDE treatment, and is most effective in inhibiting the hemagglutination caused by certain of the unheated A-prime viruses isolated in 1951. The heat stability of β inhibitor in untreated serum was not clearly stated by Smith *et al* (1951). Because of its similarity to certain heat sensitive inhibitors, the β inhibitor will be described more fully in the section devoted to heat labile inhibitors.

Different strains of influenza virus may vary widely in their sensitivity to these inhibitors (Hilleman and Werner, 1953; Jensen *et al.*, 1957). Many of the Type A influenza viruses (Far East or FE strains) isolated in 1957 were extremely sensitive to nonspecific inhibitors in heated sera (Jensen, 1957). Because the effect of inhibitor on virus is similar to that of antibody, and because sera often contain amounts large enough to lead to erroneous results, it is necessary to destroy the nonspecific inhibitors by some treatment of the sera. This can be accomplished by employing preparations of the receptor-destroying enzyme (RDE) of *Vibrio comma* (Anderson *et al.*, 1948), trypsin (Sampaio and Isaacs, 1953), or periodate (Burnet and Lind, 1954). The type of treatment depends on the virus strain being studied and on the inhibitors present in the sera to be tested. In the early work with inhibitor-sensitive FE influenza strains, treatment of sera with M/90 potassium periodate removed all nonspecific substances which inhibited hemagglutination by these viruses. Treatment with trypsin or cholera filtrates was ineffective in removing FE strain inhibitors. However, sera treated with periodate alone, although free of inhibitors active against FE strains, still contained inhibitors for such strains as A/Denver/1/57 and B/GL/54 which could be destroyed by treating the sera with trypsin. The current procedure recommended by the Communicable Disease Center (USPHS) utilizes both trypsin and periodate when sera are to be tested with inhibitor-sensitive FE and other influenza strains (Jensen, 1958).

Agglutination of certain vertebrate red blood cells is characteristic of all members of the myxovirus group and the hemagglutinin

of influenza virus is not unique in its susceptibility to nonspecific inhibitors in animal sera. Nonspecific serological reactions have been observed in hemagglutination studies with mumps and Newcastle disease virus (Wenner *et al.*, 1952a; Wenner *et al.*, 1952b). Hemagglutination of chicken erythrocytes by these viruses was inhibited by a component of normal human and rhesus monkey sera. This nonspecific inhibitor was only partially inactivated by heating at 62°C for 30 minutes but was destroyed by acetone, chloroform, trypsin, papain, and sodium periodate. The cross reactions observed in hemagglutination inhibition tests with mumps and Newcastle disease viruses and sera from persons convalescent from mumps may have been caused by nonspecific inhibitors in the sera rather than by antigens shared by these viruses.

In a recent report (Goldfield *et al.*, 1957) of hemagglutinins associated with certain human enteric viruses, nonspecific inhibition was encountered with both human and animal sera. The authors were unable to find a reliable method for removing the inhibitors and interpretation of the results of hemagglutination inhibition tests was difficult.

It is unfortunate that many of the heat stable (antibody-like) inhibitors described above and summarized in table 1 have not been adequately described. Thus there remains considerable doubt as to whether antibody, perhaps elicited by heterogenetic stimuli, may be involved in certain of the above instances. Perhaps it would be advisable to search among microorganisms other than viruses for antigenic relatives which could provide the immunogenic stimulus.

Lipid Inhibitors

A variety of fatty acids, lipids, soaps, detergents and other surface active agents have been shown to inactivate certain viruses (Begg and Aitken, 1932; Pirie, 1935; Smith, 1939; Stock and Francis, 1940; Burnet and Lush, 1940; Findlay, 1943; Theiler, 1957) but the virucidal effect of lipids in serum was first noted by Casals and Olitsky (1947). They found that lipid fractions obtained from certain normal sera either by acetone-ether or by hot alcohol extractions were capable of reducing significantly the titers of certain neurotropic viruses. Serum lipids from mice, hamsters, rabbits and horses were active against the viruses of St. Louis, Russian Far East and Japanese encephalitis. There was no correlation between the change in surface tension and the degree of viral inactivation.

In 1948 Utz investigated the effect of serum lipids on three strains of psittacosis virus. No virus inactivation could be demonstrated by normal whole serum but lipid fractions of the same sera decreased virus titers up to 100,000-fold. The virus inactivating property appeared to be in the lecithin-like and cephalin-like fractions of the serum. Egg yolk extracts and commercial lecithin, prepared in concentration equal to that expected in serum extracts, failed to exert any inactivating effect. Utz (1949) extended these studies to include four strains of influenza virus and one strain of Newcastle disease virus. A lecithin-like fraction of serum from seven different animal sources was found capable of inactivating these viruses. The extent of inactivation was similar to that previously observed with psittacosis virus. There was no change in hemagglutination titer with this inactivation nor was there any detectable "Francis inhibitor" in the lecithin-like fraction.

In an early report on hemagglutination by arborviruses Sabin and Buescher (1950) observed that normal human, rabbit, monkey and mouse sera were capable of inhibiting hemagglutination by the Japanese encephalitis virus. The inhibitory titers of normal sera were sufficiently high and variable that the hemagglutination-inhibition test could not be used as serologic evidence of infection with this virus until some means was found to remove the inhibitor without affecting the specific antibody. This heat stable inhibitor was not affected by treatment with periodate. Chloroform extraction removed 95 percent of the inhibitory activity and did not affect the antibody titer. As hemagglutinins were demonstrated among other arborviruses, additional nonspecific hemagglutinin inhibitors were found in normal sera. Chanock and Sabin (1953), in a study of the hemagglutination of the St. Louis encephalitis virus, reported that inhibitor for this agent was only partially removed from serum by chloroform in a manner that successfully removed the inhibitor for hemagglutination with the Japanese encephalitis virus. However, precipitation and extraction with acetone provided a fraction which was practically devoid of inhibitor but contained all the specific antibody. This method was effective against inhibitors for several viruses. Casals and Brown (1954) were able to eliminate nonspecific inhibitory substances by slow filtration of serum through Seitz pads. A more practical method for removing nonspecific inhibitors was introduced by Clarke and Casals (1955).

Sera were treated with small amounts of the clay mineral, Bentonite, which had been shown by previous workers (Nikkila and Oker-Blom, 1952) to selectively remove lipids from sera without significantly lowering the gamma globulin content. Following Bentonite treatment, certain sera developed the property of agglutinating chick red blood cells. This problem could be eliminated by adsorbing the Bentonite-treated serum with 10 percent washed chick cells. In addition to the simplicity of this method and its adaptability to small volumes of serum, Bentonite treatment increased the titers of some sera 2- to 4-fold over the Seitz filtration method.

An attempt has been made to summarize these reports in table 1.

Heat Labile Inhibitors

Neutralization Enhancement. Virus neutralization by specific antibody, unlike immune bacteriolysis, does not require the participation of complement. Partly for this reason, partly as a carry over of habits developed in viral complement fixation tests and partly to eliminate heat labile neutralizing factors, sera are customarily inactivated by heat prior to their use in neutralization tests. The effects of heat labile serum components in contrast to heat stable factors have therefore largely escaped investigation. Because the effects of this group of substances may be easily avoided, their importance in laboratory diagnosis of virus disease is minimal compared with heat stable factors. However, from a biological standpoint, they may have considerable importance in host defense mechanisms, as a tool for study of virus properties, and may eventually be exploited therapeutically.

Although heat labile components (including complement) are not required for virus neutralization, in some cases they may enhance the effect of specific antibody. Gordon (1925) reported a marked reduction in the virus neutralizing power of a fresh anti-vaccinia rabbit serum on heating. Activity was restored by the addition of fresh guinea pig serum. However, in experiments with two other samples of vaccinia immune rabbit serum the virucidal capacity was not lessened by heating and the addition of complement had no effect. Mueller (1931) showed that the neutralizing power of fresh antiserum against Rous sarcoma virus was enhanced by an hour's incubation at 37°C with fresh unheated guinea pig complement. In 1945, Morgan studied the effect of complement on the

neutralization of Western equine virus. It was shown that the neutralizing capacity of human and rabbit antisera was enhanced by the addition of fresh guinea pig complement. This enhancement was dependent on incubation at 37°C and did not occur when antisera-virus mixtures were held at 4°C. A serum stored in the frozen state had greater neutralizing capacity than the same serum kept at 4°C. Sera stored at 4°C could be restored to full activity by the addition of complement. Heating at 56°C for 30 minutes decreased the neutralization titers of antisera but, following this treatment, the addition of fresh guinea pig serum did not restore activity. These observations were confirmed by Whitman (1947). However Whitman was able to show reactivation of heated serum by the addition of fresh normal monkey and guinea pig sera. It was also found that the neutralizing capacity of an unheated immune serum drops more precipitously on dilution than that of heated serum. Throughout this work the enhancing component of normal animal serum is spoken of as an "accessory substance" rather than as complement. Dozois *et al.* (1949) verified the ability of fresh normal serum to enhance the neutralization of Western equine virus by heated immune serum and confirmed the thermolabile nature of this factor in fresh normal serum. The 2nd, 3rd and 4th components of complement were apparently required for neutralization enhancement. Either the 1st component was not necessary or was required only in minute amount.

Leymaster and Ward (1948) reported that a component of fresh serum, presumed to be complement, was found to exert a marked potentiating effect on the neutralization of mumps virus by heated immune serum. The neutralizing titer of inactivated immune human and monkey serum was enhanced by the addition of fresh normal sera of the same species (Leymaster and Ward, 1949). Heating at 56°C for 30 minutes destroyed the potentiating effect of fresh normal sera and reduced the neutralizing capacity of fresh immune sera. Adsorption of a serum with an antigen-antibody precipitate to remove complement also resulted in decreased neutralizing activity.

Sabin (1950), in a study of Dengue viruses using the mouse intracerebral neutralization test, found that neutralization by heated immune serum was enhanced by the addition of fresh normal rabbit, guinea pig or human sera. This nonspecific, complement-like, heat labile accessory substance produced the maximum inactiva-

TABLE 1

Spectrum of Activity of Viral Inhibitors Found in Normal Animal Sera

Type of Inhibitor	Chemical Nature	Viruses Affected		References
HEAT STABLE (56° C/30 min.)	Protein or probably protein	Adenoviruses		21, 42, 63
		Enteroviruses	Poliovirus	9, 21, 34, 45, 46, 59, 61, 83, 87, 97, 102, 103
			ECHO	21, 43*, 101
		Arborviruses	Yellow fever	14, 15, 30, 32, 64, 69
			Japanese E†	64
			St. Louis E	64
			West Nile	64
	Mucoprotein* Francis (α) inhibitor Others with nature unknown	Myxoviruses	Influenza	4, 5, 16, 18, 24, 37, 38, 48, 50, 51, 56, 57, 58, 72, 73, 88, 92, 93
			Mumps	112, 113
			Newcastle disease	112, 113
	Lipid	Myxoviruses	Influenza	106
			Newcastle disease	106
		Arborviruses‡		19*, 20, 23*, 25*, 86*
		Psittacosis		105
HEAT LABILE		Myxoviruses	Mumps	67, 68
			Newcastle disease	53
(56° C/30 min.) 1. Neutralizing 2. enhancing	Proteins and protein complexes	Arborviruses	Western equine E	28, 74, 116
			Dengue	85

HEAT LABILE		**Myxoviruses**	**Mumps**	**67, 68**
			Newcastle disease	**53**
(56° C/30 min.)			Western equine E	28, 74, 116
1. Neutralizing enhancing	Proteins and protein complexes	Arborviruses	Dengue	85
			Simbu	111
			Wyeomyia	94
		Vaccinia		44
		Rous sarcoma		75
		Lymphocytic choriomeningitis		82
		Measles		1
		Variola		70
		Herpes		39
2. Inactivating	Proteins and protein complexes (Chu, β inhibitors, properdin)	Myxoviruses	Influenza	11, 12, 24, 41, 47, 88, 92*, 93*, 95, 108*
			Mumps	41
			Newcastle disease	8, 41, 53, 54, 62, 110
		Arborviruses	St. Louis E	2
			Murray Valley E	2
			Wyeomyia	94
		Vaccinia		13, 27, 29
		Variola		70
		Herpes		33, 39
		Bacteriophage	(*E. coli*)	107
			(Staph)	100

* Inhibitor affecting hemagglutinin primarily
† E = encephalitis
‡ Including Japanese, St. Louis, Russian S.S. encephalitis, and others

tion of sensitized virus only after incubation *in vitro* for 2 hours at 37°C. The enhancing effect was not observed when the mixtures were inoculated without prior incubation.

Pollikoff and Sigel (1952) observed that the neutralizing activity of immune serum against lymphocytic choriomeningitis virus was reduced by heat inactivation. These authors recommended the use of unheated serum in routine diagnostic studies with this virus.

In a recent report on the immunologic relationship between measles and distemper virus (Adams and Imagawa, 1957) it was found that the neutralization of measles virus by canine distemper antiserum was enhanced by the addition of fresh guinea pig serum.

Weinbren *et al.* (1957) in characterizing Simbu virus, a hitherto unknown agent isolated from mosquitoes in Tongaland, observed that a thermolabile factor was present in both monkey and guinea pig serum which enhanced the activity of homologous antibody. When the neutralizing capacity of immune monkey serum was reduced by heat inactivation, its full potency could be restored by the addition of unheated nonimmune guinea pig serum.

Some reports dealing with the effects of normal serum on viral neutralization fail to indicate enhancement by heat labile factors. Howitt (1934) found that fresh guinea pig complement had no effect on the neutralization of Western equine virus (Californian strain) by horse antiserum. Florman and Enders (1942) reported that fresh normal rabbit serum did not enhance the neutralization of vaccinia virus by rabbit antiserum. Fox (1949), in studies with *Rickettsia orientalis*, observed no detectable effect on the degree of neutralization when immune guinea pig serum was inactivated or when fresh guinea pig serum was added to the system containing heated immune serum.

This survey suggests that viruses are not equally affected by heat labile neutralization enhancing factors in normal animal sera. Strain differences may account, at least in part, for the variations in results obtained by different investigators. The test system employed together with the particular combination of sera used may have influenced the outcome of investigations dealing with heat labile neutralization enhancement.

Virus Inactivation. Heat labile factors present in normal animal sera are capable of inactivating certain viruses in the absence of specific antibody. Douglas and Smith (1930) observed that normal rabbit blood possessed some degree of virucidal activity against vac-

cinia virus. This viral inhibitor was a thermolabile constituent of the serum. Enders (1949) found that fresh rabbit and cat sera, added to small amounts of vaccinia virus, reduced the size and number of pocks which subsequently developed on the chorioallantoic membrane of chick embryos. In 1951 Briody *et al.* reported the presence in normal guinea pig serum of a labile principle capable of neutralizing the egg infectivity of vaccinia virus. This nonspecific inhibitor was inactive against vaccinia hemagglutinin. Sera could be stored at −60°C with no loss of inhibitory activity but rapidly deteriorated when held for a few hours at 4°C. The rapid decline of nonspecific virucidal activity on dilution contrasts with the gradual loss of specific antibody activity. Since fresh guinea pig serum contains a high level of complement, studies were carried out to determine if this component might be responsible for the virucidal action. Adsorption of guinea pig serum with an antigen-antibody complex, which removed the hemolytic activity of complement, did not appreciably reduce the virucidal activity of the serum. This inhibitor of vaccinia virus was not present in detectable amount in the serum of mice which led the authors to postulate that this heat labile factor might be responsible for the difference in susceptibility of mice and guinea pigs to vaccinia virus following intranasal inoculation. In mice intranasal instillation of virus leads to a systemic infection whereas a similar route of inoculation in guinea pigs gives rise to an infection which is confined to the lungs.

McCarthy and Germer (1952) demonstrated what appeared to be two heat labile factors in guinea pig, rabbit and human sera active against variola virus. One factor enhanced specific neutralization of this agent by heated immune sera, the other neutralized the virus in the absence of specific antibody. These factors appeared to be independent of each other and were not correlated directly with the hemolytic complement titer.

Smithburn (1952) reported similar findings in serological studies with Wyeomyia virus. Thermolabile factors were present in the sera of nonimmune rhesus monkeys which could inactivate this agent and enhance the protective effect of antibody.

In 1953, Geller and his colleagues reported that herpes virus injected intravenously into human volunteers was not consistently recoverable from the peripheral blood. A nonspecific, complement-like, heat labile factor which had marked antiviral properties was demonstrated in the plasma of one individual. The test system em-

ployed made it impossible to decide whether the heat labile plasma factor demonstrated against herpes virus was virucidal by itself or whether it served to enhance the effect of neutralizing antibody present in the plasma.

The presence of heat labile inhibitors in normal serum may, on the one hand, serve to increase the sensitivity of specific neutralization tests, or to create false positive reactions in serologic tests. The investigator must decide in individual situations if enhanced specific neutralization by use of fresh serum is desirable in the light of possible disadvantages in reduced specificity. The relative advantages and disadvantages will depend on the nature of the virus, sera and test used.

In 1948, Howitt *et al.* reported the presence of neutralizing antibodies for Newcastle disease virus (NDV) in the serum of certain human patients with influenzal-like and neurologic disorders. Attempts to isolate NDV from these patients were unsuccessful. Subsequently (Howitt, 1950) it was shown that human serum and sera from normal monkeys, rabbits and guinea pigs also neutralized NDV. Heating at 56°C for 30 minutes destroyed the virucidal activity of all sera except those from specifically immunized animals. Hemolytic complement appeared to be necessary for the virucidal activity of normal sera. The neutralizing capacity of immune serum was decreased by inactivation and could be restored by the addition of fresh normal serum. Bang *et al.* (1950) also demonstrated a heat labile neutralizing substance for NDV in all human sera tested. However, reduction of the hemolytic activity of complement by adsorption of serum with an antigen-antibody complex did not remove the inhibitor.

In an extensive study devoted to characterizing a heat labile component of normal serum which interfered with the activities of certain myxoviruses, a thermolabile substance was demonstrated in human, guinea pig and rabbit sera which neutralized the infectivity of mumps, Newcastle disease virus and influenza A and B viruses (Ginsberg and Horsfall, 1949). The labile component of these sera and of mouse serum also inhibited the hemagglutination of chicken erythrocytes by these viruses. Treatment of serum with an heterologous antigen-antibody precipitate did not remove the thermolabile component nor was the complement activity markedly affected by quantities of virus sufficient to adsorb all the inhibitory activity. It

was concluded that the labile serum component was distinct from hemolytic complement.

Smith and Westwood (1948) described heat labile inhibitors of hemagglutination by live influenza virus in sera of mice, rabbits and guinea pigs in addition to Francis type inhibitors which were present in other species as well. Guinea pig serum, which was particularly effective, was also shown to neutralize virus infectivity. Similar inhibitors were found in human sera.

In 1951, Smith *et al.* described an inhibitor of influenza virus hemagglutination in rabbit serum which was not destroyed by RDE or by heating at 56°C for 30 minutes following RDE treatment. The inhibitor was more active against living A-prime strains isolated in 1951 than against standard reference viruses. This substance was called β inhibitor to distinguish it from heat stable α (Francis type) inhibitors described previously. Since β inhibitor is more active against living virus and is not destroyed by RDE, it may be considered as having characteristics in common with heat labile inhibitors. It is possible that treatment of serum with RDE may induce heat stability of β inhibitor by destroying or altering another serum component which combines with β inhibitor under the influence of heat. The authors did not describe the heat stability of this inhibitor prior to treatment with RDE.

Chu (1951) described a heat labile factor in normal mouse serum which inhibited hemagglutination and neutralized the infectivity of Type A influenza virus. This nonspecific substance inhibited live and heated virus to approximately the same titer. The inhibitor could be destroyed by treatment of mouse serum with crude filtrates of *Vibrio comma*, crude trypsin, or kaolin but not purified cholera RDE, crystalline trypsin or periodate. In the presence of calcium ions, the inhibitor formed a stable complex with active virus but was not destroyed by it. This inhibitor had greater activity against unadapted than against mouse-adapted virus. Adaptation of virus strains to mice or passage of virus in chick embryos in the presence of normal mouse serum led to the appearance of variants which were resistant to the inhibitor. This normal mouse serum factor has been termed "Chu inhibitor" by many workers. Sampaio (1952) analyzed normal sera from six animal species for the presence of Francis and Chu inhibitors. Mouse serum which is rich in Chu inhibitor is devoid of Francis inhibitor. Ferret and fowl sera which contain high titers of Francis inhibitor show negligible Chu in-

hibitor activity. Rabbit and guinea pig sera, however, apparently contain both inhibitors. It should be noted that heat labile inhibitors may escape detection in sera which have high titered specific antibody and/or inhibitors of the Francis type.

Briody *et al.* (1955) reported the sensitivity of egg-adapted lines of Type A-prime and Type B influenza virus to the hemagglutinin inhibiting and neutralizing activity of a heat labile inhibitor present in ox serum. Adaptation of these strains to mice was accompanied by the development of resistance to the inhibitor. The neutralizing substance was referred to as β inhibitor in this study in deference to earlier reports (McCrea, 1946; Smith *et al.*, 1951) of nonspecific serum constituents, distinct from Francis inhibitor, which inactivated influenza virus. The properties of β inhibitor are not unlike those of the inhibitor described by Chu (1951) and the two are considered identical by Brans *et al.* (1953).

A similar thermolabile inhibitor of influenza virus was described by Smorodintsev (1957). This factor was present in fresh serum of normal white mice and, like Chu inhibitor, was active against influenza strains avirulent for mice but had no effect on mouse pathogenic strains. Inhibitor-sensitive strains developed increased resistance when adapted to mice.

The use of tissue culture techniques for the study of influenza viruses may be complicated by the presence of nonspecific inhibitors in the animal serum used in the maintenance of tissue cells. The influence of normal serum inhibitors on Types A, A-prime and B influenza viruses in monkey kidney cell cultures was studied by Henry and Youngner (1957). A thermolabile substance, capable of neutralizing the cytopathogenic effects of influenza A-prime strains was demonstrated in rabbit, guinea pig and calf serum. This factor was not active against Type B or mouse-pathogenic A strains and was believed to be identical to Chu inhibitor. Since only heat labile factors were capable of neutralizing influenza virus in monkey kidney cell cultures, heat inactivation of all sera used in tissue culture systems was emphasized.

An inhibitor of Newcastle disease virus, present in normal human and rabbit serum, was studied in detail by Karzon (1956). This nonspecific viral inactivating substance (VIS) was destroyed by heat (56°C for 30 min.), trypsin, streptokinase, ethylenediaminetetraacetate and homogenates of various animal tissues. Destruction of VIS by these methods did not release infectious virus after the

VIS-virus union had taken place indicating that the virus was irreversible inactivated by VIS. A comparison of the characteristics of VIS with the β inhibitor (Smith *et al.*, 1951) of influenza virus revealed that β inhibitor was more stable on storage at 4°C than VIS. Ox serum with a high titer of β inhibitor failed to inactivate NDV infectivity. Whereas β inhibitor-resistant strains of influenza virus can be obtained by a single passage of sensitive virus in the presence of inhibitor, a VIS-resistant strain of NDV was not produced following 20 serial passages with rabbit serum VIS.

The studies cited suggest a general similarity among the heat labile inhibitors in the serum of warm blooded species. The inhibitors may differ somewhat in degree of heat stability and spectrum of activity, but in general, have many characteristics in common (virucidal activity, neutralization enhancement, and stability to RDE, trypsin and periodate) when these aspects are tested experimentally. It may be prudent, at the present time, to regard these as a single virus inhibitory complex with properties which may differ slightly among species.

The Properdin System. A previously undescribed serum euglobulin, properdin, was discovered by Pillemer and his associates (1954) in the course of experiments on the nature of complement. Properdin, in the presence of complement and magnesium ions, participates in such activities as the lysis of bacteria (primarily gram negative species) and certain abnormal red blood cells, destruction of *Toxoplasma gondii* by specific antibody and the inactivation of certain viruses. The bactericidal aspects of the properdin system (properdin, complement and Mg^{++}) were reviewed recently by Skarnes and Watson (1957) who suggested that properdin is synonymous with normal or natural antibody. For a more general review of the properties of properdin the reader is referred to articles by Isliker (1956) and Wedgwood (1958). Properdin may be removed from serum by treatment with zymosan (a yeast cell wall preparation), bacterial cell walls, and a variety of high molecular weight polysaccharide complexes of bacterial and mammalian origin (Pillemer, 1956, Pillemer *et al.*, 1957). The higher activity of the latter indicates that they may be similar to the natural substrate of properdin although the mechanism of activity is at present unknown. Properdin, in serum, is destroyed by heating at 56°C for 30 minutes although it is more stable in purified form.

The assay of properdin (Pillemer *et al.*, 1956) depends on its

property of specifically inactivating C'3 (in properdin deficient serum, RP) in the presence of zymosan at 37°C.

The properties of the heat labile serum component described by Ginsberg and Horsfall (1949) resembled those of the properdin system. Further studies (Wedgwood *et al.*, 1956) indicate that Newcastle disease virus is inactivated by the properdin system and established the identity of the nonspecific heat labile inhibitor of Ginsberg and Horsfall with the properdin system. Virucidal activity was found to be temperature dependent and required the presence of magnesium ions and complement. The amount of available properdin in serum is reduced in the virucidal reaction indicating that properdin and virus may interact in much the same way as properdin and zymosan. Ten to fifty per cent of the activity of the inactivated virus hemagglutinin could be restored by the removal of magnesium ions with a chelating agent.

Herpes simplex virus is inactivated by a heat labile factor in normal rat serum (Finkelstein *et al.*, 1958). Inactivation of herpes virus by the normal rat serum factor is temperature dependent and requires the participation of complement and magnesium ions. Inhibitory activity is removed from serum by treatment with zymosan at 17°C and can be restored by the addition of purified properdin. The results are entirely consistent with the hypothesis that the heat labile inhibitor of herpes virus in normal rat serum is the properdin system. Eight strains of herpes virus from diverse sources (superficial lesions, fatal encephalitis and mouse and egg adapted laboratory strains) were affected similarly. Attempts to develop inhibitor resistance by selection and passage of surviving virus were unsuccessful. The study suggested a possible role of the properdin system in natural resistance to herpetic infection in man. A more extensive report of these observations is in preparation.

The Chu inhibitor (described above) bears a resemblance to the properdin system (Wagner, 1955). The antihemagglutination property of adult mouse serum for unadapted influenza A virus was completely removed by treatment of the serum with zymosan.

In a survey of the virucidal activity of normal rat serum (Allen *et al.*, 1957) it was revealed that St. Louis and Murray Valley encephalitis viruses were moderately inhibited by a heat labile factor. Agents not affected included polioviruses (Types 1, 2, and 3); adenovirus (Type 4); Coxsackie viruses (B3 and B5); ECHO (Types 6, 8, 9, and 12); vaccinia (WR) and LCM (WE) (Allen *et al.*,

1958). These observations confirm the reports of Ginsberg (1956) with adenoviruses and Ledinko and Melnick (1953) with poliovirus. The authors were unable to duplicate the results of Briody *et al.*, (1951) with vaccinia. Strain differences may have accounted for this discrepancy.

Van Vunakis and her associates (1956) reported that *Escherichia coli* bacteriophage was inactivated by the properdin system *in vitro*. Staphylococcus phage (typing strain no. 53) is inactivated by a heat labile factor in normal rabbit serum which may be removed by adsorption with zymosan at 17°C (Sulkin *et al.*, 1957). Presumptive evidence was presented that properdin may be involved in the inactivation of bacteriophage *in vivo*. Later studies by us showed that the rate of bacteriophage clearance in rabbits was correlated with the properdin level.

The participation of the properdin system in natural defense mechanisms is implied in a striking series of parallelisms between properdin level and the state of resistance. However, there is, as yet, no conclusive evidence of a direct nature that properdin is involved in natural resistance. The studies of Landy and Pillemer (1956) are strongly suggestive that properdin is involved in resistance of mice to infection with gram negative pathogens and those of Southam and Pillemer (1957) and Rottino and Levy (1957) suggest a relationship in malignant disease.

Definitive studies on the role of the properdin system in virus diseases are lacking. An approach to the problem has been made (Sulkin *et al.*, 1957) with bacteriophage clearance studies in relation to properdin levels. These studies are being continued using bacteriophage and herpes virus as models of non-replicating and replicating viruses, respectively.

Conclusion

Rapid advances in methodology in recent years have placed us in an era of etiological discovery in the field of virology. In establishing an etiologic relationship between a newly isolated agent and a given disease the investigator is faced with many technical problems not the least of which is the subject under discussion. We have attempted a synthesis of present knowledge regarding the properties and importance of factors in normal serum which may modify fundamental attributes of certain viruses, infectivity and hemag-

glutinating capacity. The unwary will be confused by the similar effects of certain of these "non-specific inhibitors", especially the heat stable types, and specific antibody. Suitable controls will in many instances prevent erroneous interpretation of laboratory findings. These would include the judicious use of heat and chemical treatment to eliminate the non-specific inhibitor. In cases where "antibody" to a given agent is found in an unnatural host, systematic effort must be directed at finding the specific antigenic stimulus. In its absence, there does not appear to be any conclusive means of distinguishing the specific from the non-specific although correlative evidence may be derived from the application of a number of tests; complement fixation, hemagglutination-inhibition and neutralization, the localization of the inhibitor in a serum fraction associated with antibody, and evidence that it is not affected by treatments which inactivate known inhibitors.

The discovery of the properdin system has provided impetus to those who would study the role of non-specific inhibitors in natural resistance mechanisms. There is a marked similarity between some of the heat-labile inhibitors described in this review with the properdin system and the identity of a few non-specific inhibitors with the properdin system has been established. It remains to be seen whether others described by various investigators are sufficiently similar to the properdin system to be considered identical or whether there may be a family of "non-specific" inhibitory systems which may play important roles in natural resistance. It is not unlikely that the properdin system may be responsible for several of the reported examples of neutralization-enhancing factors. Such neutralization enhancement might be analogous to the synergistic action of the properdin system and antibody in the destruction of *Toxoplasma gondii*. Investgators should continue to focus attention on the study of the relationships of the non-specific inhibitors to natural resistance to establish if these are laboratory artifacts or significant factors which may be exploited in the control of virus diseases. As additional information becomes available about the chemical and physical nature of viruses, we shall eventually be able to establish the mode of action in many cases of non-specific inhibition and to apply this knowledge.

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PROCEEDINGS, TEXAS BRANCH OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

ABSTRACTS

Of Some of the Papers Read at the Spring Meeting
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Antibody Response to the Rous Sarcoma Virus. Lois Mendle and Morris Pollard, Department of Preventive Medicine and Public Health, The University of Texas Medical Branch, Galveston.

Rous sarcoma virus (RSV) neutralizing antibody was demonstrated 2–3 weeks after tumor development in 17 of 30 (56 per cent) chickens moribund from the effect of RSV. Of 18 tumorous chickens inoculated with RSV in an oily adjuvant, 17 (94 per cent) had RSV antibodies in their serums collected approximately 3 days after the appearance of a tumor. At this time RSV antibody appeared in eleven per cent of chickens inoculated with RSV alone. Inoculation of RSV in an oily adjuvant resulted in an accelerated antibody response in a greater percentage of inoculated chickens than in controls with virus alone.

Production of Opalescence in Egg Yolk Media by Staphylococci as an Index to Bacteriophage Typability. Charles D. Graber, Major, MSC, US Army Surgical Research Unit, Brooke Army Medical Center, Fort Sam Houston, Texas.

Phospho-lipase activity of coagulase positive staphylococci proved 100 per cent correlated with phage typability in the case of 76 aureus strains encountered in a pediatric study of unbilical stump flora. Coagulase negative albus, citreus and aurantiacus varieties, all phospho-lipase negative, did not type. Coagulase negative strains of aureus, some of which were egg yolk positive, failed to type. Coagulase positive aureus strains, that had lost their carotenoid pigment by loss variation mutation, were phospholipase positive and phage

typable. It is suggested that this might represent a method for delimiting aureus strains which have recently mutated.

Toxin Versus Toxoid Competition for Diphtheria Antitoxin. Jimmie Lee Flume and V. T. Schuhardt, Department of Bacteriology, The University of Texas, Austin.

Experimental quantitation of the apparent reversal of neutrality of diphtheria toxin-antitoxin mixtures by addition of toxoid was undertaken, employing the Fraser intracutaneous technique of toxin-antitoxin assay. Mixtures containing one Lr/100 dose toxin and varying amounts of antitoxin were made and allowed to stand one hour before the addition of toxoid. When the toxin was barely neutralized the addition of one antigenic equivalent of toxoid sufficed for reversal of neutrality of the mixture, whereas with overneutralized mixtures, up to four equivalents of toxoid were necessary and such reversal was not constant. Barely neutral toxin-antitoxin mixtures when incubated three hours required more than one equivalent of toxoid for reversal, whereas overneutralized mixtures were not reversed. Prolonged incubation of toxin-antitoxin-toxoid mixtures prior to injection resulted in some reversal in the presence of excess antitoxin, but this reversal was not as great as that shown with shorter incubation. Stabilization due to secondary reactions between TA_{2-3} complexes may account for some of the time related effects, but this does not appear to be the complete explanation.

Isolation and Serology of Azotobacterphage. James T. Duff and Orville Wyss, Department of Bacteriology, The University of Texas, Austin.

A procedure was described for isolating azotobacterphage from soils obtained from scattered geographical areas. A modified Burk's nitrogen free medium was used and the natural azotobacter flora present in the soil samples was permitted to serve as the test strain. For this reason, starting cultures were not enriched by addition of phage sensitive strains of azotobacter. One liter flasks containing 50 ml. of media were seeded with 1 gm. of soil and incubated on a shaker at 33°C for 4 days. Phage titers of 1×10^8 particles/ml. were obtained. The indicator strain for phage assays was *Azotobacter agilis* O. Based upon differences in plaque morphology, 6 phages were isolated. Antisera were prepared in rabbits against 4 of the phages and 3 phages were serologically unrelated. Host specificity

tests were undertaken to define further the identity of the phages and to ascertain the range of host infectiveness. A preliminary survey of azotobacter strains available in this laboratory showed that all strains were lysed by one or more of the 6 phages, and that the phages appeared to form 4 patterns.

Catalase Activity of Serratia marcescens *Strains.* J. H. Sessums and Robert P. Williams, Baylor University College of Medicine.

Conventional Warburg technique with H_2O_2 was employed to measure activity of the parent red strain, an orange, and two white mutants. One white mutant formed red pigment when grown with the orange. The other white mutant (WNF) did not form pigment under any conditions. All strains demonstrated activity when grown at 27°C, although WNF showed only slight response. When grown at 37°C, where no pigment formed, all strains capable of producing pigment retained high activity, but WNF had no activity. These facts suggest that catalase activity and ability to form pigment are associated. Other data indicated that all organisms possessed cytochromes. Therefore we propose that pigment is produced after porphyrin is formed, but before the prosthetic group links with protein to form catalase. Cytochrome synthesis would remain intact.

Isotopic Labeling of a Water Soluble and Diffusible Pigment Produced by Serratia marcescens. I. L. Roth and R. P. Williams, Baylor University College of Medicine.

To elucidate the biosynthesis of the water soluble pigment produced by *Serratia marcescens*, strain 125, the organisms were grown in separate bottles of liquid glycerol mineral salts media, each containing 12.5 microcuries of either acetate-1-C^{14}, acetate-2-C^{14}, glycine-1-C^{14} or glycine-2-C^{14}. After purification of the pigment by paper chromatography using a 2, 6, Lutidine: Water system (5:3 v/v) the pigment was eluted from the paper and the activity of the pigment was determined using a thin windowed G. M. tube. It was found that the organism utilized each of the radioactive carbon atoms in the production of the pigment, however, the exact levels of the labeling by each compound has not been as yet determined.

The Use of a Card Sorting System in the Identification of Bacteria. Carl D. Heather, Texas State Department of Health, Austin.

The system described will point out and yield information on certain closely related biochemical groups of organisms. The key sort system described is not original in principle; a similar system is produced commercially by the McBee Company of San Antonio. Holes were punched around the margin using a master card as a guide. It is essential that punching be done with care so that all cards match. The individual holes or groups of holes represent the reactions as follows: Morphology (rod or coccus) Gram stain, motility, nitrate reduction, indole production, Hydrogen sulfide production, gas from carbohydrates, catalase, fermentation of dextrose, lactose, and sucrose. An individual card is prepared for each organism. When a hole is notched out, it means that the organism in question is positive for that particular reaction or characteristic. An unnotched hole represents a negative reaction. An adjacent hole is allowed for each reaction and indicates an unknown or unlisted reaction. When cards are to be sorted for a given series of reactions, they are first lined up so that the holes match; the steel wire sorting needle is passed through the holes representing some particular reaction and the cards lifted up. Positive organisms fall from the deck; negative ones remain on the needle.

The Use of Antibiotics in Screening Strains of Bacteria for Lysogeny. E. D. Rosenblum and J. L. W. Jackson, Department of Microbiology, The University of Texas Southwestern Medical School, Dallas.

A sensitive method for screening for lysogeny is presented. Strains are tested for lysogeny by directly spotting broth cultures of antibiotic sensitive strains on agar plates containing the antibiotic and which are seeded with an indicator strain. The strains used as indicators include both naturally occurring antibiotic- resistant strains and antibiotic-resistant mutants of sensitive strains. This method gave a tenfold higher plaque count with strains of *Staphylococcus aureus* than the usual method of spotting supernatants of centrifuged cultures. In a preliminary study with various enterobacteria, one-third of the cultures screened by the antibiotic method were detected as lysogenic or colicinogenic, and yielded a high number of heterologous reactions, *i.e.* intergeneric and interspecific.

Properdin Levels in Epidemic Influenza. Richard A. Finkelstein and S. Edward Sulkin, Department of Microbiology, The University of Texas Southwestern Medical School, Dallas.

Paired serum samples from 14 patients with serologically proven influenza were assayed for properdin by a modification of the zymosan assay technique proposed by Pillemer *et al.* Changes in properdin level were detected in nine of the patients. Of these, eight had lower titers in the acute sera, while in one patient with Type B influenza the reverse change was noted. The frequency with which changes in properdin level occurred in these patients is statistically significant. These results are in contrast with observations on a similar series of paired sera from healthy subjects in which properdin levels were constant. The significance of these findings and the possibility that they may be more apparent than real was discussed.

Evaluation of Liquid Soaps for Antibacterial Activity. William A. Nolte, Department of Microbiology, The University of Texas Dental Branch, Houston.

Four different brands of liquid soap were tested for residual antibacterial effect following a standard hand wash technic. Three brands containing G-11 (hexachlorophene) referred to as T, S, A, and the fourth soap, nongermicidal, containing cocoanut oil base, referred to as Y, were used exclusively in hand washing during the test period.

The standard hand wash technic consisted of washing the hands with a measured amount of soap and water, collecting the rinse in a sterile basin and determining the microbial numbers in the rinse by the plate count technic. Two per cent serum was added to petri plates to inhibit the bacteriostatic effect of G-11 carried over in the rinse water platings.

After two days' use, soap T reduced the microbial flora of the hands 98.9 per cent, soap S, 67.5 per cent and soap A, 82.8 per cent. Soap Y did not demonstrate any residual degerming effect.

The prolonged antibacterial effect of soaps containing G-11 is shown in other tests. By comparison it was demonstrated that six consecutive washings with a nongermicidal soap are required to reduce the microflora of the hands to approximately the level that can be effected by a soap containing G-11.

Polio Virus Types Found in 1957. Carolyn Gabriel, Thelma D. Sullivan, and J. V. Irons, Division of Laboratories, State Health Department, Austin.

Fifteen polio strains were isolated from feces inoculated in HeLa tube cultures. Identification was based on results obtained in neutralization tests employing type specific polio antisera. Eleven of the isolates were Type I, one was Type II, and two were Type III. One specimen contained both Type I and Type III virus.

The majority of the polio isolates were obtained from children under five years old. Studies on contacts of suspected polio cases yielded polio as well as other viruses. Fifty cases of paralytic polio in patients who had received two Salk shots or more were reported to the Texas State Communicable Disease Control Division. Specimens tested on seventeen of these cases yielded two strains of poliovirus and two non-polio cytopathogenic agents. Two hundred twenty-two paralytic, non-vaccinated cases were reported. Nine polioviruses were obtained from specimens on fifteen of these cases. Four polio isolates were found in specimens from non-paralytic cases.

Studies on Staphylococci Isolated from a Hospital Population. I. Phage Types and Antibiotic Sensitivity Patterns of Strains from Personnel. Joseph A. Bass, Jo Anne Holzman, and Harriet M. Felton, Departments of Microbiology and Pediatrics, The University of Texas Medical Branch, Galveston.

Complete phage typing and antibiotic sensitivity patterns of coagulase positive strains of *S. aureus* isolated from the anterior nares of personnel associated with the John Sealy Hospitals revealed a much higher incidence of the 80/81 strain in patients and in personnel in close association with patients than in employees in other areas. Conversely 44A and Group III strains were isolated with greater frequency from non-patients. The distribution of other types was general. Forty-six per cent of all strains were non-typable.

Multiple resistance to antibiotics occurred in 84 per cent of 80/81 strains, 30 per cent of non-typables, 15 per cent of Group III strains, and 10 per cent of Group I. However, of the multiple resistant strains isolated, 49 per cent were non-typable, 39 per cent were 80/81's, whereas the remaining 12 per cent were evenly distributed

among various other types. These studies were conducted as a baseline for comparing the incidence of various types in the anterior nares of a hospital population to their frequency of occurrence in clinical specimens in subsequent studies.

Studies on Staphylococci Isolated from a Hospital Population. II. Phage Types and Antibiotic Sensitivity Patterns of Strains from Patients. Jo Anne Holzman and Joseph A. Bass, Department of Microbiology, The University of Texas Medical Branch, Galveston.

S. aureus isolates from Clinical Pathology Laboratories were phage typed over a period of approximately one year following a general survey of hospital personnel. Groups I, II, and IV appeared relatively infrequently in both cases and carriers. Group III and 44A organisms, although isolated frequently from carriers, were involved in only a small percentage of known lesions. However, of the Group III isolates, 42 per cent were found to be resistant to two or more antibiotics. On the other hand, 80/81 strains appeared twice as frequently in established infections (50 per cent) as in patient carriers, and four times as frequently as in the general hospital poplation. Multiple resistant, non-typable strains were isolated from 13 per cent of lesions. Although incidence of the latter strains in lesions was the same as in carriers, they were considered to represent a potential pool of cross-infection from which an epidemic strain could arise.

Molecular Weight in Relation to the Susceptibility of Pseudomonas Aeruginosa *to Antibacterial Compounds.* W. F. Verwey, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey, and The University of Texas Medical Branch, Galveston.

Strains of *Pseudomonas aeruginosa* are relatively resistant to most of the commonly used therapeutic agents and antiseptics. Over a period of several years about 5,000 organic compounds of extremely diverse chemical structure have been studied to determine their inhibitory activities against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and several other organisms. Study of these data indicated that the relative resistance of the pseudomonas to antibacterial activity was quite marked in comparison to that of the staphylococcus. Five hundred seven compounds showed a finite degree of inhibitory activity against both the pseudomonas and the

staphylococcus and therefore activity ratios could be calculated for these compounds. These varied widely. It was found, however, that when the inhibitory ratios were plotted against molecular weight, a significant trend was apparent which indicated that as molecular weight increased the relative resistance of the pseudomonas organisms also was greater. Since this trend was apparent in this group of highly diverse compounds, these results suggest that the sensitivity of the pseudomonas to antibacterial substances may be influenced by some factor related to molecular size. It is suggested that differences between the ability of the staphylococcus and the pseudomonas to take in larger molecular weight compounds may be at least a partial explanation of the results that were obtained.

MORPHOLOGICAL DESCRIPTION OF SELENOID BODIES

MANUEL CUADRA[a]

In a previous paper we have established that Selenoid Bodies are stromas of erythrocytes (1), and that they are derived from these when smears of lipemic blood are made.

In the present article we will consider the morphology of these bodies: these studies are of interest because they are related first with the structure of normal erythrocytes and secondly with the investigations of selenoid bodies in pathological conditions, a subject on which we are working at present.

Materials and Methods

Examination of blood smears stained by a new procedure (1, 2). The samples of blood were taken from normal subjects two to three hours after taking breakfast consisting of one piece of bread, one cup of milk and 30 grams of butter.

In some cases the blood smears were put in a moist chamber (a Petri dish with some filter paper soaked in distilled water) immediately after making them, that is, without letting them dry. They were kept in the humid chamber for varying times, from a few minutes to one hour or several hours; or the dessication was retarded several minutes by putting the slide with the smear parallel with another one (the spreader) a very short distance apart. A few times the smears were made on previously heated slides in order to obtain the effect opposite to that of the moist chamber, that is, a quick dessication.

Results

The following is the description of selenoid bodies as they appear in dry and stained smears, that is, as almost bidimensional corpuscles. Schilling (3) affirms having seen them in wet preparations.

Selenoid bodies have the general appearance of crescents; we distinguish in them two fundamental parts, the "lunula" and the "vesicle" (Fig. 1). The old staining methods showed the "lunula" clearly and the "vesicle" vaguely. Our staining method (1, 2) shows them definitely, making such names as "half moons" and "crescents" obsolete, as they disregard one of the

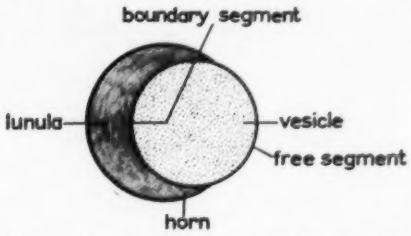

FIG. 1. Schematic drawing of a selenoid body.

elements of what we now call "selenoid bodies"; this denomination, which comprises both parts, should be therefore preferred. We have denominated "lunula" the most visible part of these bodies, which resembles the illuminated part of the moon when it is in its first quarter. The term "lunula" is used in mathematics to designate the figure of this shape which is formed by two intersecting circles; this definition can be applied to selenoid bodies, for we see in them two intersecting circles of different diameter. The bigger circle is incomplete and forms the convexity of the lunula; the smaller circle is complete and corresponds to the vesicle, the round and less visible part of the selenoid bodies which we compare with the non-illuminated part of the moon when it is in its first quarter. This part we call "vesicle" because it has the properties of a vesicular formation, such as a round shape, tendency to become a perfect circle, propensity to grow and to burst; it has a finely granular content. We do not find suitable the name "vacuole" (Schilling, 3), because it implies vacancy; we will later describe vacuoles inside the lunula, these are circular spaces without any interior structure.

Selenoid bodies look slightly oval in most cases; the long axis cuts the lunula transversely through its widest part, and the short axis passes through its horns. The diameter of the bodies of normal persons is of about 15 microns; in thin smears it seems larger than in thick ones. The size of these elements keeps the same uniformity as among normal erythrocytes. In anisocytosis, as in cases of hemolytic anemia, there is the same abnormality in the selenoid bodies (Fig. 2); gigantic specimens of diameters up to 40 and 50 γ may be found in the same conditions; this is the case in Carrion's disease (human bartonellosis) during the phase of hematic regeneration (intense reticulocytosis).

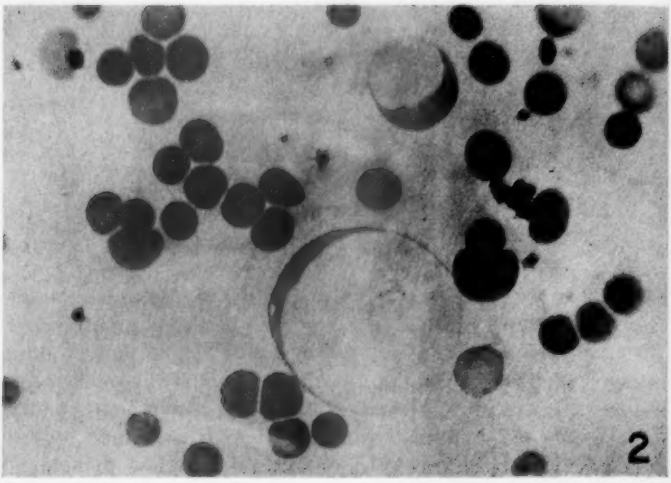

FIG. 2. Selenoid bodies showing marked variation in size. Chronic hemolytic anemia.

The size of selenoid bodies depends mainly on the size of the vesicle; the area of the lunula remains fairly constant in all bodies, large or small, while it is the vesicle which varies in dimensions. We therefore consider that when a selenoid body grows, the growth takes place at the expense of the vesicle, the lunula being distended without any alteration of its area, though it may sometimes decrease a little.

In the lunula we must consider its convexity, its concavity, its surface and its horns (Fig. 1). In smears of normal blood the lunula may be of the following kinds: 1. Uninterrupted edges and surface ("whole lunula"); 2. Broken edges and surfaces ("ravelled lunula"); 3. Eroded surface as if chiseled or eaten by a moth ("moth eaten lunula") (Figs. 3, 4 and 5). Vacuoles may appear in all these types of lunula; these vacuoles are devoid of any interior structure. Types 2 and 3 may coexist (mixed forms).

The percentage of selenoid bodies with "whole lunulae" is greater in thick smears than in thin ones; next in number are those with "ravelled lunulae," the "moth eaten lunulae" being very rare. In thin smears the proportion between types 1 and 2 is usually in favor of the former though frequently this condition

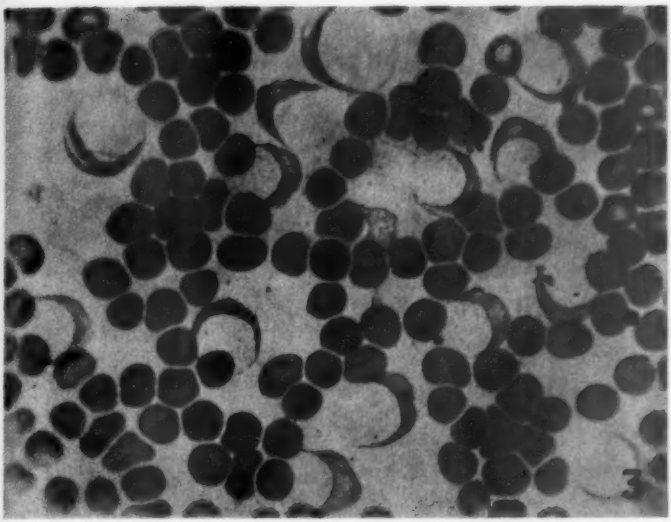

FIG. 3. Selenoid bodies with "whole lunulae" (uninterrupted edges and surface.

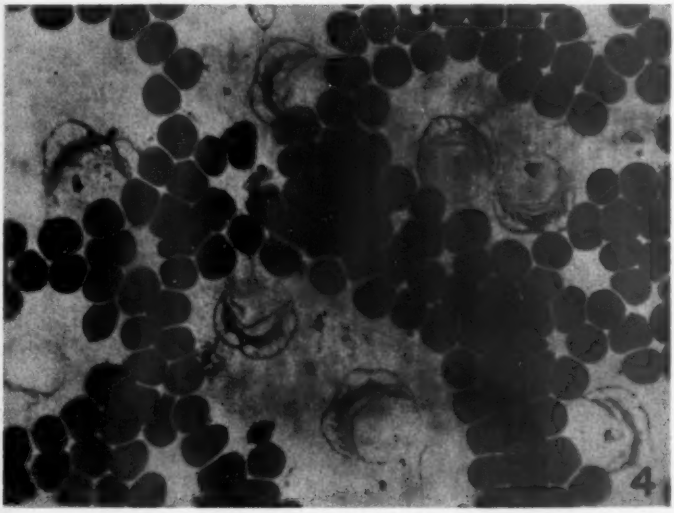

FIG. 4. Selenoid bodies with "Ravelled lunulae" (broken edges and surface).

is inverted. The following is a typical "formula" of a thick smear: 90 per cent selenoid bodies with "whole lunulae," 10 per cent with "ravelled lunulae" and 0 per cent with "moth eaten lunulae." In thin smears the formula would be: 40 per cent with "whole lunulae," 58 per cent with ravelled lunulae, and 2 per cent with moth eaten lunulae."

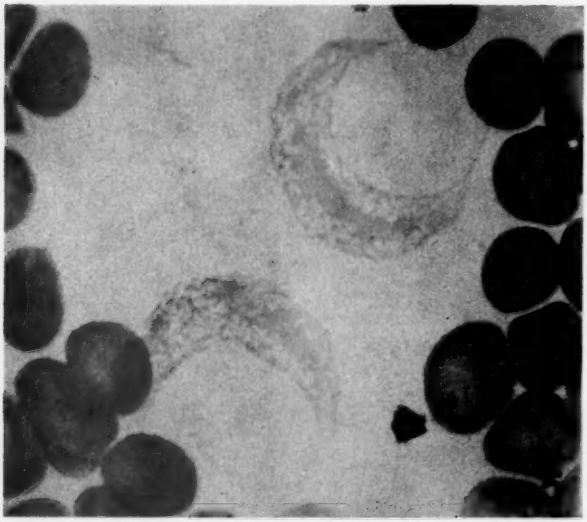

FIG. 5. Selenoid bodies with "moth eaten lunulae" (eroded surface).

The convex edge of the lunula may be regular or waved; it is generally regular when the vesicle is big and keeps it distended (sickle-shaped lunula) (Fig. 6), and waved when the vesicle is small (clover-shaped lunula) or when it bursts while growing and the force which kept it distended ceases to act (Fig. 7). The surface of the lunula with waved edge may be wrinkled and crossed by striations that start at the incision of the waves, and advance even to the vesicle.

In the moist chamber the lunula frequently adopts the form of a ring which completely surrounds the vesicle (Fig. 8).

When under some circumstances the vesicle breaks, always at the free segment, the lunula retracts frequently until it finally becomes round.

The vesicle tends to be circular and is placed in the concavity of the lunula. It is sometimes crossed by striations which start from the lunula. If we consider it as flat, one side of it is in contact with the concavity of the lunula ("boundary segment") and the other seems girded by a filiform prolongation of the lunula, like a loop ("free segment"). The vesicle may vary greatly in size, from very small to enormous; the latter case is seen in hemolytic anemias.

The vesicle undergoes important morphological changes in the moist chamber; first, its size is usually greater, and secondly, its shape tends to be perfectly circular. It will sometimes burst because of its growth, always at the segment girded by the filiform prolongations of the lunula (free segment); the tips of the broken loop are clearly seen and the granular content of the ves-

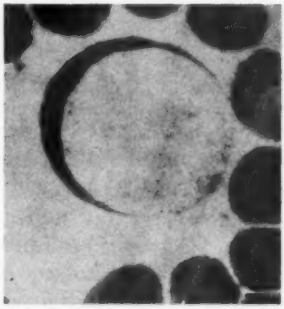

FIG. 6. Selenoid body with sicle-shaped lunula.

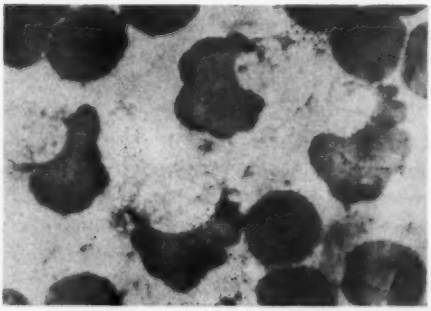

FIG. 7. Selenoid bodies with their lunula showing waved edges; consequence of the rupture of the vesicle.

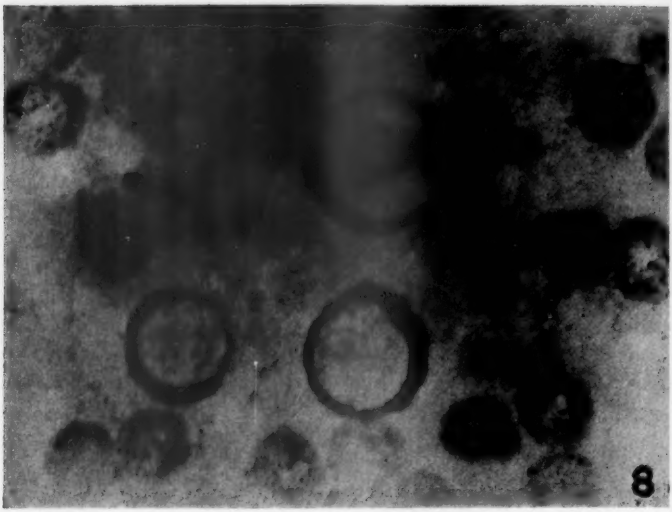

FIG. 8. Selenoid bodies showing appearance of rings (wet chamber).

icle is set free. We have already stated that as result of this rupture the convex edge of the lunula comes away, the immediate cause being the disappearance of the intravascular tension.

The blood smears of subjects who have ingested much fat and who have assimilated it well show a large number of selenoid bodies and a gamut of transitional forms between normal erythrocytes and these elements. If the blood smear is made on slides heated to 37° or 38° C, their number is still greater. The transitional forms are the following: erythrocytes with sinuous edges, of a pale dull red color, with branches which, starting from the periphery, grow inwards, making them look as if they were wrinkled or as if its structure would have softened up; and swollen erythrocytes (pseudomacrocytes), big, of a pale dull red color. We think that these two elements represent the first stages in the formation of selenoid bodies. Later these elements lose their hemoglobin content (expelling stage), apparently through one of the poles, which is the one in which the vesicle develops, while the lunula is formed in the opposite pole. The hemoglobin comes out as a red cloud which spreads, and often contains, especially in smears made on previously heated slides, a blue-black powder;

this powder probably corresponds to the substance that makes up the nucleus like corpuscle present in many erythrocytes, denominated by us "pseudonormoblasts" (1).

Transitional selenoid bodies, with their hemoglobin half way expelled, are frequently seen in the edges of the smears; this fact may be explained because drying begins at the edges of the smears, stopping the evolution of the bodies at an earlier stage. These transitional bodies do not stain well on account of their hemoglobin content, that is to say, the lunula and the vesicle look blurred.

In patients suffering from hemolytic anemia (intense reticulocytosis) we have detected upon staining with brilliant cresyl blue and then with our method (1), reticulated selenoid bodies, in which the reticulum was sometimes placed in the lunula and sometimes in the vesicle. Nucleated selenoid bodies are originated from the normoblasts; this nucleus is usually placed in the vesicle.

Discussion and Summary

In the present study we have made a morphological description of selenoid bodies indicating all possible variations. It follows from this that the bodies are constituted of two essential and inseparable parts: the "lunula" and the "vesicle," different in morphology, structure and properties; this suggests that the normal erythrocytes could be made up of two parts also: the marginal zone, from which the lunula would be derived, and the central zone (colorless area), corresponding to the vesicle.

The vesicle is being able to swell (Schilling 3), distending the lunula and sometimes causing the rupture of the wall of the corpuscle, always at the "free segment." The lunula instead is characterized by its elasticity, because when the vesicle bursts (thereby dissipating the force causing its distention), it retracts and becomes rounded. All these forms are easily observed in blood smears. Our observations reinforce Schilling's findings; the studies of this author indicated that the "central vacuole" (we call it "vesicle") of selenoid bodies (Halbmondkörper in German) is produced by the swelling of the "vitreous body" (central colorless area of erythrocytes); and the pink crescent in the margin (we call it "lunula") produced by hemolysis of the stroma (3).

The formation of selenoid bodies is a phenomenon occurring

in vitro which involves the lipids of the blood, and the friction of erythrocytes against the surface of the slides while making the smear. Lipids have not been demonstrated in the vesicle. It is logical to admit that abnormal selenoid bodies may be produced by constitutional or acquired anomalies of erythrocytes, or by variations of the quality of the lipids of the blood. The investigations we are now making show us that selenoid bodies may, in certain pathological conditions, be completely unlike those found in normal bloods; these findings will be published elsewhere.

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[a] From the Facultad de Medicina de Lima, and the Hospital Dos de Mayo, Lima, Peru. Accepted for publication August 30, 1958.

THE EFFECT OF PLANT EXTRACTS ON EGG CULTIVATED TUMOR TISSUE

USHA C. DALAL,[a] ALFRED TAYLOR AND GEORGE F. MCKENNA[b]

It has been observed that there is not a single chemical agent which will completely inhibit the growth of or destroy tumor tissue without causing undue disturbances to nontumor tissue. Various compounds do adversely affect certain malignant growths but at the same time are so toxic to nontumor tissues as to be of limited value in clinical work. A number of compounds occurring naturally in fungi and other plants are successfully used in the therapy of many diseases associated with pathogenic bacteria. Plants especially would seem to be worth investigating in this respect, since the plant kingdom has yielded so many potent medicines in the past. A number of cancer chemotherapy experiments with plant extracts have been previously reported (Taylor *et al.*, 1952; Taylor *et al.*, 1956; McKenna *et al.*, 1958). The plant collecting data and some information as to chemical constituents is available (Wall *et al.*, 1954a, 1954b, 1955).

This report is concerned with data obtained from the work done with 96 plant extracts including 91 species of plants[c]. The results obtained with some of the plants, using a different testing technique, have been reported (McKenna *et al.*, 1958).

Experimental Procedure

The plant extracts were tested against egg cultivated tumors. The method used has been described (Taylor *et al.*, 1952, 1956; Taylor, 1953a, 1953b, McKenna *et al.*, 1954). Embryonated eggs were yolk sac-inoculated through the air sac on the 4th day of incubation with a saline suspension of dispersed tumor tissue. Eggs were implanted with C_3H mouse mammary carcinoma which grows with relative uniformity in every inoculated egg. The tumor implanted in the eggs by the yolk sac method attains the size of 0.2 gm. at 8 to 10 days of incubation. The various extracts were introduced over the area vasculosa of 11-day tumor bearing eggs by a method described (Taylor, 1950). This material injected in this manner passes into the blood stream of the chick embryo and is carried to the yolk sac

TABLE 1

Plant Extracts Inhibiting the Growth of C3H Mammary Carcinoma in Tumor Bearing Eggs (48-Hour Test Period)

Family	Plant	USDA Accession No.	No. of Exps.	No. of Eggs	Temp. of Incubation ° F.	Dosage, mg. of Dried Material	Survival, Control = 100	Average Growth for Test Period, Control = 100	
								Tumor	Embryo
Anacardiaceae	Loxopterygium huasango	2701	2	64	99	3.64	115	20	109
Anacardiaceae	Pistachia chinensis	6103A	2	66	99	4.09	84	33	124
Anacardiaceae	Pistachia chinensis	6103B	2	66	99	5.95	112	23	95
Anacardiaceae	Pistacia lentiscus	4153	6	202	99	7.7	106	10	122
Anacardiaceae	Pistacia lentiscus	4153	3	100	104	7.7	98	11	95
Apocynaceae	Funtumia sp.	2186A	2	67	99	9.82	89	39	94
Apocynaceae	Funtumia sp.	2186A	1	39	104	9.82	92	7	80
Combretaceae	Conocarpus erecta	2128	1	26	99	12.5	61	18	112
Combretaceae	Conocarpus erecta	2128	1	26	104	12.5	44	22	107
Compositae	Bigelowia nudata	4990	3	110	99	10.2	89	40	99
Compositae	Bidens pilosa	4873	3	101	99	4.90	116	31	120
Compositae	Boltonia diffusa	4340	2	73	99	6.94	71	37	104
Compositae	Cirsium altissimum	4538	1	33	99	9.80	72	42	90
Compositae	Gaillardia pulchella	3730	1	31	99	2.17	58	33	118
Compositae	Mikania scandens	4590	1	34	99	11.1	88	33	99
Compositae	Silphium compositum	4257	2	70	99	4.1	87	42	118
Compositae	Spilanthes americana	4029	4	143	99	7.58	89	45	101
Compositae	Spilanthes americana	4029	1	36	104	7.58	99	0	100
Caprifoliaceae	Viburnum acerifolium	4853	2	52	99	10.4	72	13	109

Caprifoliaceae	Viburnum acerifolium	4853	2	52	99	10.4	72	13	109
Caprifoliaceae	Viburnum acerifolium	4853	1	40	104	10.4	117	52	89
Ebenaceae	Diospyros kaki	1850	2	66	99	3.48	65	8	102
Euphorbiaceae	Sapium sebiferum	1410	1	23	99	22.3	60	0	107
Leguminosae	Acacia spadicigera	1303	1	27	99	12.9	85	15	104
Leguminosae	Adenanthera microsperma	2954	3	102	99	12.5	85	37	100
Leguminosae	Adenanthera microsperma	2954	1	37	104	12.5	62	5	81
Leguminosae	Gleditsia triacanthos	1400	3	93	99	6.42	61	10	102
Leguminosae	Gleditsia triacanthos	1400	2	72	104	6.42	56	43	93
Leguminosae	Leucaena glauca	2198	1	29	99	10.5	50	32	104
Leguminosae	Piscidia mollis	1335	1	23	99	4.80	60	15	121
Moraceae	Humulus japonicus	4254	2	67	99	7.93	104	29	133
Moraceae	Humulus japonicus	4254	1	29	104	7.93	50	25	121
Oleaceae	Forsythia sp.	1812	1	31	99	9.8	58	5	74
Oleaceae	Forsythia sp.	1812	1	31	104	9.8	58	0	120
Palmae	Phoenix roebelenii	2648	1	38	99	7.46	54	44	102
Palmae	Pseudophoenix vinifera (petiole)	3659	4	149	99	7.81	112	41	97
Pinaceae	Callitris quadrivalis	2403	2	63	99	5.81	93	45	108
Pinaceae	Callitris quadrivalis	2403	1	41	104	5.81	114	45	86
Rosaceae	Agrimonia gryposepala	4531	1	33	99	10.6	95	22	115
Rosaceae	Duchesnea indica	3807	1	34	99	10.3	104	39	118
Rutaceae	Atalantia citroides	4714C	2	76	99	2.35	85	54	112
Rutaceae	Atalantia citroides	4714C	2	76	104	2.35	69	7	110
Sapindaceae	Sapindus utilis	2480	2	55	99	11.1	82	30	115
Solanaceae	Cestrum parqui	4379B	1	30	99	5.81	107	45	97

implanted tumor. Numerous experiments have demonstrated that the embryos of egg injected with saline solution according to this procedure will continue their growth, survival and development exactly as if they were untreated. Some of the experiments were carried out at 104° F. as described by Taylor and Williams (1956).

Groups of about 15 eggs were utilized for the testing of each extract. A group of 20 or more controls received H_2O. The effect of the plant fraction was based on weight differences in the tumors and embryos of the experimental eggs as compared with the controls.

Results

The results are summarized in tables 1 and 2. Table 1 lists 32 plant species which inhibited tumor growth 50–100 per cent during a 48 hour test period. There was very little effect on the growth of embryos. Some extracts were more effective in inhibiting tumor growth at 104° F. Some of the test substances stimulated tumor growth. A few of these extracts have the same effect at 104° as at 99° F. Table 2 lists all the plant extracts having negligible effects on tumor growth.

TABLE 2

Plant Extracts with Little or No Effect on C3H Egg Cultivated Mammary Carcinoma (48-Hour Test Period)

Family	Plant	USDA Accession No.
Amaryllidaceae	Agave expansa	1003
Anacardiaceae	Loxopterygium huasango	2651
Anacardiaceae	Pistachia terebinthus	6127A
Anacardiaceae	Pistachia terebinthus	6127B-1
Compositae	Cacalia atriplicifolia	4244
Caprifoliaceae	Symphoricarpos orbiculatus	4640
Palmae	Butia nehrlingiana	2146
Palmae	Latania commersonii	2028
Phytolaccaceae	Limeum aethiopicum	3517
Rutaceae	Phellodendron sp	1363
Sapindaceae	Alectryon subcinereum	2650
Sapindaceae	Dodonaea viscosa	3003

Discussion

None of the plant extracts processed to date have given results which promise practical application to cancer chemotherapy. Some of the extracts are effective up to a certain extent and need further attention. The data indicates the anti-cancer possibilities of the

naturally occurring compounds present in the higher plants of the plant kingdom and encourage further work in this field.

Summary

Extracts from 91 plant species were tested for their effect on the growth of egg-yolk sac cultivated mouse mammary tumor. Extracts from 32 species inhibited tumor growth 55–100 per cent as compared with H_2O treated controls. Three species of the family *Anacardiaceae* gave extracts which inhibited tumor growth 67–90 per cent. Extracts of 8 species of the family *Compositae* inhibited tumor growth 55–70 per cent. Five species of the family *Leguminosae* gave extracts which inhibited tumor growth 63–95 per cent.

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FACTORS IN THE ETIOLOGY OF CHLORPROMAZINE JAUNDICE: AN EXPERIMENTAL STUDY

IVAN E. DANHOF*

The increasing number of reported incidents of hepatic involvement is evidence that jaundice of an obstructive type is a major side effect complicating chlorpromazine (CPZ) administration. Although the etiology of the jaundice remains obscure, several possible mechanisms have been postulated. Pelner and Waldman (22) have suggested that CPZ-induced jaundice may be related to a suboptimal methyl group pool level based on the premise that CPZ, containing a dimethyl amino grouping, may function as a choline antagonist. It has been shown, however, that CPZ-induced jaundice can occur in apparently well-nourished individuals (13). Other possible mechanisms have been advanced. The studies of Zatuchni and Miller (29) revealed portal area infiltration by blood elements which might be related to CPZ-induced jaundice. These and other authors believe that the mechanism of the jaundice may be attributed to a hypersensitivity or allergic manifestation to the drug (7, 26, 29). This is supported both by the relative incidence of CPZ jaundice (5, 16, 20, 24, 27, 28) as well as the expression of frank eosinophilia (4, 7). The concept that the drug is hepatotoxic has been presented (29) but others (21, 22) doubt the validity of such a concept. Intrahepatic cholestasis, based on evidence obtained from liver biopsies, has been indicted as a current mechanism in the production of jaundice (7, 9, 15, 17, 19) with biliary canalicular plugging attributable to a specific increase in bile viscosity brought about by CPZ administration (9, 17). In view of the several etiological possibilities enumerated, a study was undertaken to investigate in albino rats, fed complete or choline deficient diets, the relationships between chronic CPZ administration and growth, effect on the level of peripheral eosinophils, adrenal weights, liver histology, and bile volume and viscosity including the effects of dehydration.

Methods

Series 1. Four groups of 10 male albino rats, Sprague-Dawley strain, each weighing approximately 75 to 80 gms. were marked and maintained (in animal rooms maintained at 76° F. and 40 per cent relative humidity) on measured amounts of the following diets and drugs for 20 days. Water was given *ad libitum*, but volumes consumed were measured daily. Group 1: Complete diet plus saline, intraperitoneally (i.p.), daily, in equivolume per weight as the dosage of CPZ[b]. Group 2: Complete diet plus CPZ, 25 mgm./kgm., i.p., daily, with CPZ in saline solution with a concentration of 10 mgm./cc. Group 3: Choline deficient diet plus saline, i.p., daily, as in Group 1. Group 4: Choline deficient diet plus CPZ, i.p., daily, as in Group 2. Each animal was weighed daily just prior to feeding. The amount of ration given was regulated by pairing Groups 1 and 3, and 2 and 4, so that the intake of the partner groups was approximately equal. Food was presented at approximately 12 meridian each day, while the saline or CPZ injections were given later in the afternoon o feach day. This permitted the animals (nocturnal feeders) to have access to the ration for several hours prior to the rather marked sedation which was seen to occur in the CPZ-treated animals. On the first day of the experiment, a dosage of 50 mgm/kgm. was given (the approximate 14 day LD_{50} being 75 to 100 mgm./kgm.)[c], but the sedation was effective for over 24 hours. The animals consequently were unable to ingest food; hence, with subsequent daily administration of CPZ, the dosage was reduced to 25 mgm./kgm.

The basic experimental choline deficient diet consisted of: sucrose, 73 per cent; vitamin-free casein, 18 per cent; Illinois salt mixture, 4 per cent; corn oil, 5 per cent. Vitamin supplementation per kilogram of diet consisted of: thiamine, 15.0 mgm.; riboflavin, 20.0 mgm.; nicotinic acid, 50.0 mgm.; pyridoxin, 10.0 mgm.; calcium pantothenate, 50.0 mgm.; biotin, 0.3 mgm.; folic acid, 1.5 mgm.; menadione, 25.0 mgm.; vitamin A, 20,000 USP units; vitamin D, 2,000 USP units; α tocopherol, 100.0 mgm.; ascorbic acid, 1.0 gm.; inositol, 75.0 mgm.; PABA, 75.0 mgm.; and vitamin B_{12}, 0.05 mgm. In addition 1.0 gm. of choline chloride was added to the basic diet and fed to the control animals and was considered to be the "complete" diet. The daily body weights and measured food intakes were determined to observe growth rates of the animals as well as to measure the efficiency of the gain in body weight with and without CPZ medication. Nose-rump lengths were also measured at sacrifice to determine if any untoward effects on trunk length were evident.

CPZ has been shown (3, 14, 17, 23) to have a profound hypothermic effect. The rectal temperatures on each animal were determined prior to and 1 hour following the administration of saline and CPZ on the 10th through the 14th days of the dietary study.

On the eighteenth day of the study, tail blood samples were taken postprandially, but prior to saline and CPZ injections, and the number of eosinophils estimated using Henneman's modification of Randolph's method (8).

At the termination of the feeding experiment, 15 hours after the last saline or CPZ injections, each of the animals was anesthetized with intra-

peritoneally administered pentobartial sodium (4 mgm./100 grams of body weight), the abdomen opened, and a sample of liver removed, blotted free of excess blood, and fixed immediately in 10 per cent formalin solution. The tissues were sectioned and strained with hematoxylin and eosin in the usual manner and sections from each animal examined.

The adrenal glands were removed, the excess fat and connective tissue removed, and each pair labelled and sealed in parafilm packets to prevent drying until weighing which was accomplished using a 500 mgm. balance. The adrenal weights were expressed in mgm./100 gms. of body weight for comparison. This parameter was measured to assess the amount of stress the diets and drug administration imposed upon the animals.

Series 2. Additional control and experimental groups of rats, consisting of 25 animals each, weighing from 110–140 grams, were maintained on the complete diet for 10 days following which the groups received for 8 days saline and CPZ, respectively, the mode of administration, dose, and volumes, in every respect similar to the aforementioned groups. At day 0 and 2 day intervals following initiation of treatment, five rats from each group were anesthetized, a laparotomy performed, the bile ducts cannulated, and bile collected for a 1½ hour period in a manner to exclude excessive exposure to air to prevent evaporative loss. The volume of collected bile was measured, diluted 1:10 or 1:20 with distilled water, and the viscosity relative to water determined using an Ostwald viscosimeter. This study was undertaken to observe any possible change in volume or viscosity of collected bile over an 8 day period with varying exposure to CPZ medication. Statistical limits of the method are given.

Series 3. The sedative response to CPZ was sufficiently profound to affect the amount of food and water ingested by the animals. To investigate the effect of the state of hydration on bile volume and viscosity, sixteen additional animals, weighing 285–325 grams, were maintained for seven days on complete diet and water *ad libitum* with food and water consumption measured daily. Half of these animals were given intraperitoneal saline and CPZ daily, respectively. During the second seven day period the animals were given food *ad libitum* (the total ingested being measured daily) but water presented was one-half of the average *ad libitum* water intake of the previous seven days. The daily intraperitoneal injections of saline and CPZ were continued during the second week. On the fifteenth day the animals were anesthetized with pentobarbital sodium, the bile duct cannulated, and bile collected for 1½ hours. The volume of bile collected was recorded and the viscosity relative to distilled water was determined by the Ostwald method.

Results

Series 1. Sedative Response to Chlorpromazine. It was noted during the early days of the experimental period that the sedative response at the 25 mgm./kgm. level of CPZ administration had a duration of 8 to 10 hours. During the latter days of the experi-

ment, however, the duration was 4 to 6 hours in length. No difference between the dietary groups was noted.

Growth. In table 1 are presented the growth data observed and calculated from the daily body weights and measured food intakes. Significant decreases ($P < 0.01$ using Fisher's "t" test) in final weights and gain per animal were observed in the CPZ-treated animals. The total gain was decreased in the CPZ-medicated groups with the greatest total gain occurring in Group 3 (choline deficient diet and saline injections). The total amounts of ration eaten by Groups 1 and 3 (saline injected) and Groups 2 and 4 (CPZ-injected), respectively, were very similar although one diet was complete and the other choline deficient. Decreases in the total ration ingested in the CPZ-medicated groups may be attributed in part to the marked sedative response induced by CPZ and the lack of interest in the environment and available ration. The sedative response may, in part, be due to the depressed metabolic rate in CPZ-medicated animals as evidenced

TABLE 1

Growth, Eosinophil Count and Adrenal Weight Data: Animal Groups on Complete and Choline Deficient Diets With and Without Chlorpromazine

Condition	Group 1	Group 2	Group 3	Group 4
No. of Animals/Group	10	10	10	10
Diet	Complete	Complete	Choline Deficient	Choline Deficient
Medication	Saline	CPZ	Saline	CPZ
Initial Wt./Animal (gms.)	78.2±1.1*	78.4±0.9	76.4±1.1	78.8±1.0
Final Wt./Animal (gms.)	132.8±2.2	112.6±1.4	134.7±1.8	122.0±1.8
Gain/Animal (gms.)	54.6±2.1	34.2±1.4	58.3±1.6	43.2±1.4
Nose-Rump Length (mm.)	167 ±3.2	158 ±2.1	165 ±1.6	157 ±2.0
Average Diet Intake (gms.) /Animal/20 Days	149.6	132.4	153.3	132.6
Average Water Intake (gms.)/Animal/20 Days	329.6	254.2	326.5	248.0
Ratio $\frac{\text{Gms. } H_2O}{\text{Gms. Ration}}$	2.20	1.92	2.13	1.87
Grams Ration/gm. Gain	2.74	3.87	2.63	3.07
Peripheral Blood—Eosinophil Count—18th Experimental Day (Count per $mm.^3$)	381 ±16.2	5353 ±185.5	367 ±18.4	4015 ±65.0
Adrenal Weight—(mgm./100 gms. Body Weight)	16.3±0.6	22.2±1.2	18.5±0.4	24.3±2.6

* $S.E. = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

by decreases in rectal temperature (Table 2). The average ingestion of H_2O was observed to be reduced in the CPZ-treated groups with the H_2O per ration ratios decreased in the CPZ-treated animals signifying a lesser intake of water per gram of ration than in the saline treated groups. Nose-rump lengths merely reflect the general depressant effect of CPZ on ingestion of food.

Eosinophils. A 10–14 fold increase in peripheral eosinophils was observed in groups medicated with CPZ both on the complete and choline deficient diets (Table 1).

Adrenal Weights. Significant increases in adrenal weights were seen in the CPZ-medicated groups (2 and 4) when compared with the saline injected groups (1 and 3) (Table 1). The choline deficient and saline group (3) also showed statistically significantly higher adrenal weights than the complete diet and saline group (1) at the 1 per cent confidence level.

Hepatic Histology. The livers of Group 3 animals appeared pale, light yellowish in color, and grossly were abnormal. whereas the livers of Groups 1, 2 and 4 had the normal deep red-brown color and consistency, and appeared macroscopically normal. Since the livers of Group 3 were grossly so indicative of severe fatty metamorphosis, no fat analyses of the livers were undertaken. In figures 1 through 4 are presented representative photomicrographs of liver sections from the 4 groups of animals. The histological interpretation of the hepatic sections is given by groups. Sections were made from the livers of each of the animals

TABLE 2

Changes in Rectal Temperature of the Rat Following Injection of Saline or Chlorpromazine

Condition and Diet	Group 1 Complete Diet	Group 2 Complete Diet	Group 3 Choline Def.	Group 4 Choline Def.
Medication	Saline	Chlorpromazine	Saline	Chlorpromazine
Rectal Temperature Prior to Injection (° C.)	*36.4±0.4†	36.4±0.8	36.3±0.8	36.2±1.0
Rectal Temperature One Hour after Injection (° C.)	36.3±0.2	31.9±0.7	36.3±0.6	29.6±1.2
Difference Rectal Temperature (° C.)	0.1	4.5	0.0	6.6

* Average values for daily measurements for 5 days (10th through the 14th days of experiment).

† S.E. $= \sqrt{\frac{\Sigma\, d^2}{N\,(N-1)}}$

and all the animals from each group were studied and the composite opinion rendered below.

Group 1: Complete diet plus saline. Sections from the livers of this group of animals show the same normal histologic pattern, the usual lobular architecture being present. There is no evidence of biliary obstruction, inflammatory change, parenchymal cell damage, or eosinophils about the portal areas.

Group 2: Complete diet plus chlorpromazine. Sections of liver from this group of animals show a normal architectural pattern similar to Group 1. In the periportal zone of the liver, the parenchymal cells show minimal granulation of cytoplasm with an occasional binuclear cell indicating regeneration. These changes are minor and as seen are often within the limits of physiological regeneration of the liver.

Group 3: Choline deficient plus saline. Sections of liver from these animals reveal marked architectural distortion by numerous vacuoles within the parenchymal fibrous cytoplasm. There is diffuse fatty metamorphosis with apparent increased severity of the process involving the central area. There is no evidence of inflammatory change or of biliary obstruction. Regeneration changes are not observed.

Group 4: Choline deficient plus chlorpromazine. Examination of the slides of this group of animals reveals no evidence of fatty metamorphosis. The architectural pattern is not distorted, and all slides have the same histological features similar to Group 2.

Series 2. Bile Viscosity. In table 3 are presented the data concerning bile volume and viscosity measured in animals who were exposed to chlorpromazine for varying periods up to 8 days all the while being maintained on "complete" diets. The standard error together with the significance of the difference of the means as tested by using Fisher's "t" test are given. Through the second day on chlorpromazine no changes from the normal in bile volume or viscosity were noted; however, with the fourth day of chlorpromazine administration and continuing through the eighth day of the experiment, the chlorpromazine injected animals showed decreases in collectable bile volume and increases in bile viscosity.

Series 3. With one-half *ad libitum* water restriction the volume of bile collected decreased while the viscosity increased (Table 4). The volumes of bile collected from the control (saline injected) animals are somewhat lower and the viscosity some-

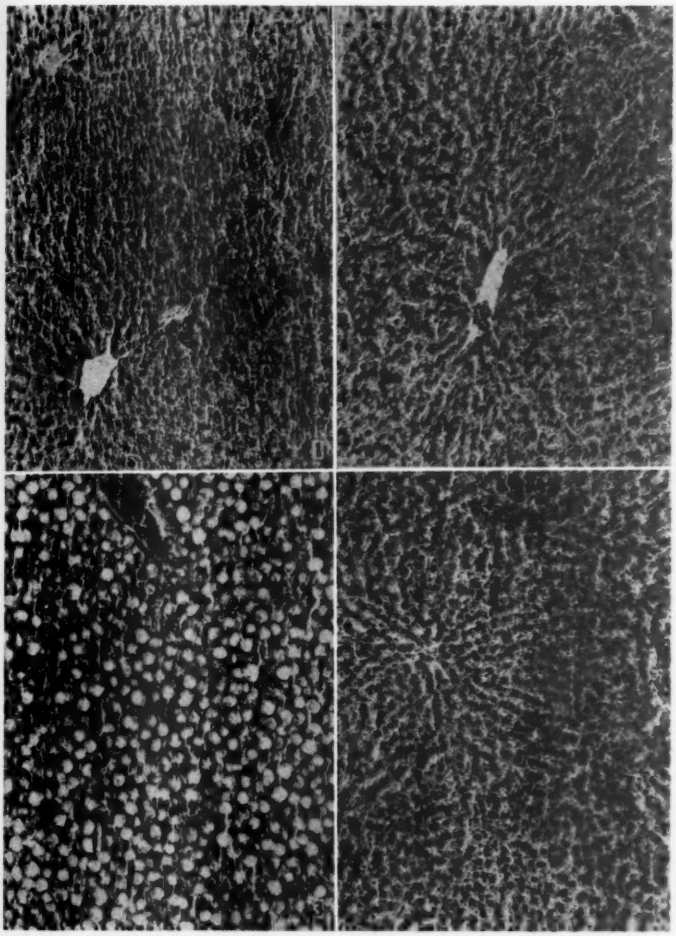

FIG. 1. An hepatic section from a Group 1 (complete diet plus saline) animal showing normal lobular architecture. X170.

FIG. 2. An hepatic section from a Group 2 (complete diet plus chlorpromazine) animal showing essentially normal liver architectural pattern. X170.

FIG. 3. An hepatic section from a Group 3 (choline deficient diet plus saline) animal showing marked architectural distortion by numerous vacuoles characteristic of diffuse fatty metamorphosis. X170.

FIG. 4. An hepatic section from a Group 4 (choline deficient diet plus chlorpromazine) animal showing architectural normality and an absence of changes of fatty metamorphosis. X170.

TABLE 3

Bile Viscosity Measurements

Day	Number of Animals per Group	Condition and Units	Group 1 Controls	Group 2 Experimentals	Significance
		Bile flow without treatment—ml. per 1½ hr. collecting period	1.3±0.08*	1.4±0.07	N.S.†
0	5	Bile viscosity without treatment —relative to water or 1.0	11.3±0.31	12.0±0.32	N.S.
2	5	Bile flow—ml.	1.4±0.07	1.2±0.05	N.S.
		Bile viscosity— relative units	13.0±0.17	12.8±0.29	N.S.
4	5	Bile flow—ml.	1.2±0.05	0.8±0.04	$p < 0.01$
		Bile Viscosity— relative units	11.2±0.56	18.1±1.31	$p < 0.01$
6	5	Bile flow—ml.	1.3±0.03	0.5±0.07	$p < 0.01$
		Bile Viscosity— relative units	10.8±0.89	23.8±0.41	$p < 0.001$
8	5	Bile flow—ml.	1.2±0.08	0.3±0.04	$p < 0.01$
		Bile Viscosity— relative units	12.2±0.79	25.8±0.32	$p < 0.001$

* S.E. $= \sqrt{\frac{\Sigma\, d^2}{N\,(N-1)}}$

† Fisher's "t" test.

what greater than comparative values of control animals in Series 1 (Table 2). With water deprivation the increase in bile viscosity is not significantly greater with CPZ medication than with saline. The reduction in the water per ration ratios decreases with the water deprivation, and is intensified in the CPZ-treated groups (compare Table 1). It should be noted that the weights of the animals under conditions of water deprivation were decreased rather markedly with the major decrease in ingestate being attributable to the water intake rather than a reduction in the amount of ration ingested.

Discussion

Sedative Responses. The duration of the sedative period following initial chlorpromazine (25 mgm./kgm.) was observed to be 8 to 10 hours and was characterized by profound disinterest in

the environment. Although the animals could be aroused relatively easily, spontaneous postural movements were almost nonexistent. The depth of depression apparently was complete within 5 minutes after the drug administration with no observable subjective difference in arousability of the animals at 10 minutes, 1 hour and 4 hours. The initial onset of depression was delayed during the latter days of the experiment as long as 20 minutes. The animals were subjectively more easily aroused, showed a greater number of spontaneous changes in position, and a decrease in duration of effects to 4 to 6 hours. These changes probably indicate a tolerance to the drug by the animals.

Growth Responses. It was observed that CPZ-medicated groups of animals showed less gain over the 20 day experimental period than those groups receiving saline injections. This is attributed to the marked depression seen in the CPZ-medicated group. The medication significantly reduced the time available for ingestion of food as well as producing a decreased interest in the environment although food was available. The decrease in the total food intakes of the CPZ groups tends to substantiate this premise. Little difference was observed in the amount of ration ingested by the saline injected groups indicating that the choline deficient diet was ingested as readily as the complete diet. With

TABLE 4

Effects of Water Deprivation on Collectable Bile Volume and Viscosity

No. of Animals	Rat Wt. (gms.)	Experiment—7 days: *Ad Libitum* H_2O Intake (ml.)	Ration Intake (gms.)	Rat Wt. (gms.)	Experiment—7½ days: *Ad Libitum* H_2O Intake (ml.)	Ration Intake (gms.)	Rat Wt. (gms.)	Bile Vol. (ml.)	Bile Viscosity
				Saline Injected Group					
8	312± 2.2*	264	128	324± 1.8	139	111	313± 2.1	.81± 0.7	17.9 ±1.1
		$\frac{\text{Ratio } H_2O}{\text{Ration}} = 2.06$			$\frac{\text{Ratio } H_2O}{\text{Ration}} = 1.25$				
				CPZ Injected Group					
8	323± 1.4	182	99	293± 1.8	91	90	264± 2.3	.33± .04	18.5 ±0.6
		$\frac{\text{Ratio } H_2O}{\text{Ration}} = 1.84$			$\frac{\text{Ratio } H_2O}{\text{Ration}} = 1.01$				

* S.E. $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

respect to the efficiency of gain, it is interesting to note that the gain in the choline deficient group was as efficient as the gain in the complete diet group. It should be noted that despite the apparent efficiency of gain in the choline deficient group, the hepatic histology supports the belief that a rather severe choline deficiency was produced in Group 3, whereas in Group 1, the diet was sufficiently complete in methyl groups to prevent any marked distortion from normal hepatic plate arrangements. With CPZ medication a decrease in efficiency of gain was observed. It would be assumed that in the saline groups without CPZ medication and the subsequent sedative response, muscular activity would be greater, and hence, the energy available for body growth (gain) would be decreased. In the CPZ-medicated groups where muscular activity would be curtailed, more energy would be available for gain; however, less gain was observed. In order to attempt an explanation, an additional parameter of CPZ action must be considered, namely, the hypothermic responses accompanying the administration of the drug at the dosage utilized in these experiments. A marked and signifiant reduction in rectal temperatures was seen to occur in the CPZ-treated animals. No differences were observed in the initial rectal temperatures between the complete and choline deficient groups. It is postulated that the depression in body temperature indicates that the metabolic rate of the drug-injected animals was decreased, and since the optimum temperature range for general enzymatic action was markedly altered, the ration utilization of the CPZ-medicated group was less efficient. Likewise, a significant portion of the sedative response may, in all probability, be attributed to the depressed nervous system function incident to the drug-induced hypothermia and hypometabolic activity.

Eosinophilia and Adrenal Hypertrophy. A marked increase in peripheral eosinophils was observed in the CPZ-medicated animals whether on the complete or choline deficient diet when compared with their saline control groups. This is interpreted to indicate a hypersensitivity to the drug. With regard to the adrenal per body weight ratios, the values indicate significant hypertrophy in the CPZ-treated groups when compared with their saline controls as well as in the choline deficient-saline group (3) when compared with the complete diet-saline group (1). That the stress of choline deficient diet alone might result

in an increase in adrenal hypertrophy might well be expected as would the additional stress of the drug administration. It is impossible, however, to reconcile the marked peripheral eosinophilia and simultaneous adrenal hypertrophy since the reciprocal relationship between peripheral eosinophils and adrenal hypertrophy (presumably indicating an increased production of corticoids) is well appreciated. That the adrenal hypertrophy was unaccompanied by the increased output of corticoids, thus permitting eosinophilia, in view of the imposed dietary and medicinal stresses, is untenable. Likewise, with increased levels of peripheral eosinophils of the magnitude observed and the fact that the increase was consistent to the animal in all the CPZ-treated groups, makes it very unlikely that the eosinophilia was not a real entity.

Liver Histology. Choline deficiency fatty metamorphosis was produced in Group 3, whereas, the normalcy of the liver in the Group 1 animals indicates sufficient completeness of the "complete" diet to forestall hepatic involvement. The CPZ-treated animals maintained on choline deficient diet also fail to show diffuse fatty metamorphosis. It is, therefore, assumed that since rigid experimental conditions were strictly maintained, CPZ protected the liver of the choline deficient animals from typical choline deficiency diffuse fatty metamorphosis. The mode of action of this CPZ protection remains obscure. Two speculative explanations might be offered. First, since CPZ contains a dimethyl amino group, it might be suspected that under the stress of choline (methyl group) deficiency the drug functions as a methyl group donor. This is not tenable since (1) chemically the donation of methyl groups from CPZ is unlikely, and (2) so-called "non-specific" protection by the thyroid depressing drug, thiouracil (1), against renal changes attributable to maintenance on choline deficient diets has been observed in animals. The protection against choline deficiency renal fatty infiltration by thiouracil may be related to protection against hepatic fatty metamorphosis by CPZ through the common action of depressed metabolic rate with the liver (possessing a somewhat higher metabolic rate than the somatic areas of the body) being able to carry out its lipid metabolic functions under CPZ-induced hypometabolism. Second, it has been shown (12) that CPZ induces a lipemia clearing effect similar to that observed with heparin ad-

ministration (25). The first mentioned authors concluded that CPZ caused a release of heparin to the blood and that the lipemia clearing effect was mediated by heparin. The mechanism of heparin action in inducing lipemia clearing has been investigated and recently reviewed (2). CPZ protection against choline deficient fatty metamorphosis may be related, then, to the lipemia clearing factor (CF) induced by CPZ and mediated via heparin. This scheme of hepatic protection by CPZ presupposes that the work of the liver is aided by CF-induced intravascular lipid cleavage and that the hepatic work of metabolizing the fatty acid-protein complex which is formed by the liberated free fatty acid bound to serum albumin (10, 11) is either independent of the methyl group pool or substantially bypassed.

It should be noted that no eosinophilic infiltration or biliary canalicular plugging was evident in the hepatic sections.

Bile Viscosity. The mechanisms producing the increase in bile viscosity and reduced bile volume with CPZ are completely obscure. In the experimental animals given CPZ no biliary canalicular plugging was evident either in the groups fed choline deficient diet or groups fed the control diet. The animals studied for CPZ effect in bile did show, however, an increase in viscosity. Increased biliary tree pressure has been reported in the CPZ-treated cholecystectomized dog (18). In view of the depressed bile volume and concomitant increase in bile viscosity, the idea that normal total solids are being excreted under CPZ administration but with a reduced amount of aqueous solvent secreted is an attractive but at present unfounded concept. The data concerning bile volume and viscosity under condition of water deprivation tend to indicate that the increase in bile viscosity is related primarily to the state of hydration of the animal. It should be emphasized that although CPZ is associated with an increase in bile viscosity, the mechanism is probably not a direct action on the liver but seems indirectly related to the volume of water intake. Interestingly, the volume of bile collected from the CPZ-treated animals is only 38 percent of that collected from the saline-injected group while the viscosities show only small differences. This is interpreted to mean that (1) the total biliary solids are either not excreted in the bile, with jaundice of a retentive nature possible, or (2) the total biliary solids formed are less than normal in

amounts and related to CPZ-induced hypometabolic action, in which case the total biliary solids could be secreted in a smaller volume with slightly increased viscosity in which circumstance jaundice would be unlikely. It must be forcefully emphasized that no definite correlation between clinical etiology of CPZ jaundice and these experimental data are warranted at this time for two reasons: (1) the transfer of data or extrapolation of results to other than this experimental species is not without potential error and (2) no signs either subjectively during the experiment or objectively by analysis of hepatic sections could be related to the state of jaundice.

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[b] Chlorpromazine was generously supplied by Smith, Kline and French laboratories, Philadelphia, Pennsylvania.

[c] Professional literature on "Thorazine," Smith, Kline and French laboratories.

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INFLUENCE OF ELECTROSHOCK STRESS ON DESOXYCORTICOSTERONE INDUCED HYPERTENSIVE CARDIOVASCULAR DISEASE IN THE SENSITIZED RAT

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Whether or not the chronic exposure of animals to non-specific stressors is capable of inducing any of the so-called "adaptation diseases" remains uncertain. Selye (9) has reported that rats sensitized by unilateral nephrectomy and an increased sodium chloride intake develop the lesions of hypertensive cardiovascular disease when subjected to prolonged exposure to cold. Since these same changes can be induced by chronic desoxycorticosterone acetate (DCA) administration, and cold exposure causes adrenal hypersecretion, it was inferred that the latter was responsible. Recently Ingle (1, 5), who disagrees with Selye's interpretation, has reported upon the occurrence of cardiorenal lesions in the sensitized cold-exposed rat, but is inclined to ascribe them to NaCl toxicity consequent to the augmented intake which characterizes such animals. Thus the divergence of opinion which currently exists is not as to the facts, but as to their interpretation.

Although cold-exposed rats drink more saline than controls, this might well be a necessary concomitant of endogenous adrenal mineralocorticoid hypersecretion. DCA treated rats offered a 1 per cent NaCl solution consume it in prodigious amounts. It seems likely that endogenous secretion of desoxycorticosterone, or a similar mineralocorticoid, would have the same result. Such a combination is known to predispose to vascular and connective tissue hyalinosis in the rat (2, 4, 6, 11, 13). Whether the adrenals play an etiologic or merely a permissive role in the pathologic changes induced in the sensitized cold-exposed rat, and whether adrenal hypresecretion or sodium chloride toxicity is the ultimate culprit, are not the most crucial questions which obtrude.

The most cogent criticism which can be leveled against cold stress is one which does not appear to have received serious consideration from protagonists of either persuasion, namely that

cold is not a non-specific stressor. Specifically it causes thyroid enlargement and increased thyroid hormone secretion (16). Thyroid hormone, in turn, is known both to potentiate the cardiovascular lesions caused by simultaneously administered adrenal steroids (10) or Vit. D (3), and also, when used alone, to produce the same lesions (8). Manifestly, then, the heart and kidney lesions encountered in cold-exposed rats may be due to cold *per se*, *to* hyperthyroidism induced by cold, to hypercorticism induced by cold, to hypercorticism induced by hyperthyrodism, or to a combination of these, and even this does not exhaust the possibilities. If cardiovascular lesions are to be used as indices of the damaging effects of non-specific stress, either some agency other than cold will have to be employed, or a way of ruling out the effects of thyroid hormone will have to be found.

In an attempt to discover a method capable of inducing chronic stress, free of the objections to cold exposure, we were encouraged to explore the possibilities of repetitive electrical stimulation. The present experiments, utilizing this principle, were designed to answer three questions; firstly, whether, in sensitized animals, stress alone was capable of provoking cardiovascular-renal disease; secondly, whether the response to small doses of DCA differed in stressed and non-stressed animals; and thirdly, whether the necessity of depriving animals of food and water during stress exposure would seriously limit the usefulness of the method.

Materials and Methods

The electrically-activated cage consisted of a rectangular frame of transparent plastic 60″ x 18″ x 9″, with a metal grid floor. Removable plastic partitions permitted having as many as 16 cages approximately 7″ x 7″ x 6.5″. The floor consisted of brass rods of 3/8″ diameter spaced at 1 cm .intervals. Alternate rods were of different polarity and, connected to a 110 V outlet through a transformer which reduced the current to 35–40 V, provided the agency through which shocks could be delivered. The desired frequency of shocks and their individual duration was arranged through contacts on an electrically driven kymograph capable of operation at various speeds. Rats were so exposed 6 days a week for 8 hours a day. Preliminary studies showed that any projection or recess in the cubicles was utilized by the rat to avoid contact with the floor, hence food and water were not provided during the period of stress. Control groups were similarly deprived whenever the experimental animals were being stressed.

Experiment I. The series consisted of six groups of female rats of the Holtzman strain. Groups 1–4 each consisted of eight animals and groups 5 and 6 of

six animals, 60–90 grams in weight. All were subjected to unilateral left nephrectomy and given 1 per cent NaCl solution to drink. Group 1 animals received 200 γ of DCA suspension daily, and were subjected to electroshock stress (ESS), shock of 6 seconds duration being given every 6 minutes. Group 2 animals received no hormone, but were subjected to ESS along with group 1 animals. Group 3 animals received 200 γ day DCA, but no ESS; and group 4 animals served as uninjected controls; both of these groups, however, were deprived of access to food and water during the period that group 1 and 2 animals were being stressed. Group 5 animals received 200 γ day DCA, and group 6 animals no hormone, both having unrestricted access to food and 1 per cent NaCl solution.

Blood pressure of each of the animals in the unanesthetized state was measured periodically by a tail plethysmograph as described elsewhere (14). Pressures above 150 mm. Hg. were regarded as hypertensive. The experiment was terminated on the 42nd day. The cardiac ventricles, kidneys, adrenals and thymus were examined, dissected out, weighed on a delicate torsion balance, and placed in fixative for histologic examination.

Experiment II. Thirty-two female rats of the Holtzman strain weighing 76–100 g. were divided into four equal groups. All were subjected to left nephrectomy and given 0.85 per cent NaCl and Purina Laboratory Chow *ad lib.*, with the exceptions noted before. Those of groups 1 and 2 each received 400 γ/day DCA by subcutaneous injection, groups 3 and 4 received no hormone. Groups 1 and 3 each were exposed for 8 hours daily, 6 days a week, to 6 second 30–40 volt shocks delivered at 6 minute intervals, during which time they and their respective controls were deprived of access to food and drinking solution. Blood pressures were taken periodically thereafter as described in the preceding experiment, and the animals killed on the 74th day. The organs were processed as in the previous experiment.

Results

Growth. In experiment 1, animals given free access to food and water at all times grew at the same rate as those with restricted intake. Similarly there was no evidence that either the time of onset or the severity of the hypertension induced by hormone treatment was affected by dietary restriction.

Mortality. In experiment 1 three animals in each of the ESS-exposed groups died before the end of the experiment, and in the second experiment one in the hormone treated stress group and five of the eight in the control stressed group did the same. Of these deaths, five were due to either extensive gastrointestinal hemorrhage or pulmonary edema and seven to spinal injury. The latter resulted when rats bit the floor rods during the passage of current and then, with the teeth engaging the rods, threw the body sideways. As the experiment progressed the DCA treated

animals became less responsive to the intensity of shock which continued to excite the controls. This was particularly true in the second experiment wherein the dosage of hormone was greater, and in which mortality of stress controls was five times as great as among the hormone treated groups receiving the same stress. DCA is known to increase electroshock threshold (15), and rats chronically treated with this hormone had less objective reaction to the shocks. Deaths among DCA treated non-stressed animals were largely due to the pathologic changes accompanying hypertension, and in controls to pneumonia.

Blood Pressure. DCA and ESS each proved capable of eliciting an elevation of blood pressure, but when given together the results were much more dramatic. The results of the first experiment were essentially the same as the second; which may be used to illustrate the efficacy of the combined treatment. By the 74th day there were only three animals surviving in the group exposed to stress alone and these had blood pressures of 166, 160 and 152mm. Hg. Of the 8 animals treated with DCA, the blood pressures ranged 130–174 mm. Hg., four being below 150 mm. Hg. and four above. All of the seven animals treated with both DCA and ESS were hypertensive, the pressures ranging 166–220 mm. Hg. In both experiments the number of animals affected and the magnitude of blood pressure elevation was greatest, and the onset most rapid, in the stressed animals receiving hormone. The data are presented in figures 1 and 2.

Thymus. In the first experiment there was no evidence that the thymus gland was appreciably affected, although the unexpectedly small size of these glands in controls suggested that perhaps these might have atrophied because of some minor disorder, thus preventing a basis for comparison. In the second experiment surviving animals exposed to stress alone were too few in number to permit analysis, but the glands from rats exposed to ESS + DCA were smaller than those from controls ($p < .001$) or from animals treated with DCA alone ($p < .01$).

Adrenals. As evidenced by increase in weight, adrenal hypertrophy was evident only in the first experiment. Here it was confined to those animals exposed to stress alone which had developed hypertension. Since this was not true of DCA treated stressed animals, it might be inferred that DCA treatment tended

to interfere with pituitary ACTH discharge, or with the action of this hormone on the adrenal cortex.

Kidneys. In experiment 1 the kidneys of the various experimental groups were not significantly larger than those of con-

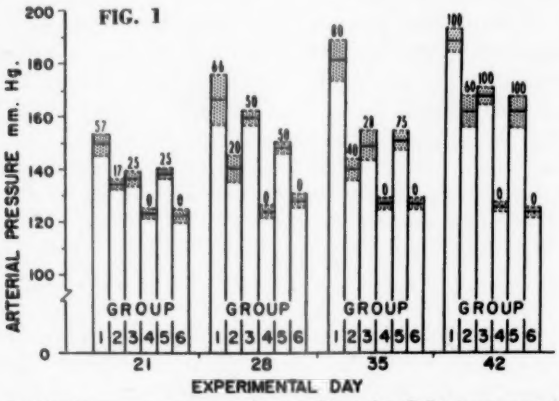

FIG. 1. Blood pressure response of rats subjected to DCA treatment (groups 3 and 5), electroshock stress (group 2); a combination of the two (group 1) and controls (group 4 and 6). The solid line at the top of each column demarcates the group mean arterial pressure, stippled area on either side delimits the standard error of the measurement, and the superscript figure the percentage of animals with pressures above 150 mm. Hg.

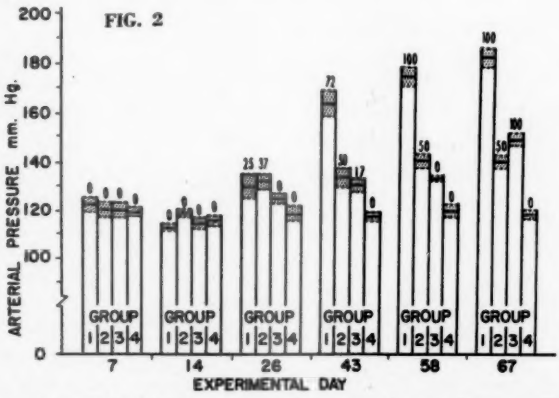

FIG. 2. Sequential blood pressures of groups of rats. Group 1, DCA + electroshock stress (ESS); group 2, DCA alone; group 3, ESS alone and group 4 controls. Other details as in figure 1.

trols. In experiment 2 however, although neither DCA nor stress alone significantly altered renal size, kidneys from animals subjected to both ESS and hormone were larger than those from controls ($p < .025$), or stressed animals ($p < .005$), whereas those from animals receiving only DCA were not.

Heart. Increased ventricular weight, presumably due to cardiac hypertrophy, characterized with one exception all hypertensive groups. Generally there was agreement between the severity of the hypertension and the degree of cardiac enlargement, hence hearts were largest in the animals subjected to both ESS and DCA. The exception was constituted by animals exposed to ESS alone in the second experiment. These were very mildly hypertensive and that only for a brief period of time, which may account for the lack of cardiac enlargement.

Cardiovascular-Renal Lesions. Renal lesions were found in varying degree and intensity in animals subjected to either ESS, DCA administration, or a combination of these in experiment 1, but not with DCA alone in experiment 2. In animals exposed only to stress these consisted just of tubular dilatation, with occasional casts, and enlargement of the cells comprising the parietal layer of Bowman's capsule. Hyalinization or necrotic changes in glomeruli or arterioles were noted only in animals treated with DCA or a combination of DCA and ESS. Cardiac lesions, consisting of either myocarditis, granuloma formation or necrotic changes in vascular walls, were likewise restricted to the latter groups. At the lower dosage of hormone, experiment 1, there was no clearcut difference among the DCA treated groups insofar as lesions were concerned. In experiment 2, cardiac and renal lesions were found only among animals exposed to both DCA and ESS. Only in this group were there animals in which the blood pressure had exceeded 200 mm. Hg., and only these showed visible lesions. The data are summarized in tables 1–2 and the histologic characteristics of the lesions in figures 3–9.

Discussion

The efficacy of ESS as a stress is evidenced both from the visible and audible reaction of the rats, and from the fact that gastrointestinal erosions may be produced with it as we and others (7) have found.

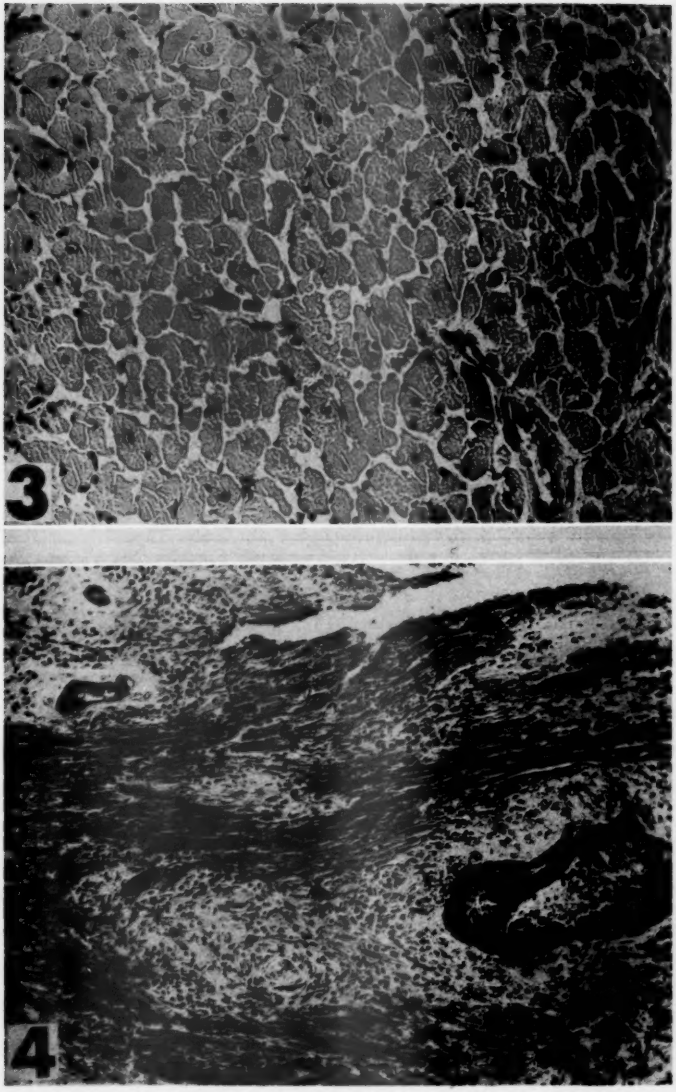

FIG. 3. Section from control heart showing normal myocardial fibres in cross section. Hematoxylin and Eosin x 260.

FIG. 4. Section from myocardium of rat given 200 γ/day DCA and subjected to ESS. Note subendothelial hyalinization of vessel walls and the surrounding granuloma tissue. Gomori's trichrome x 130.

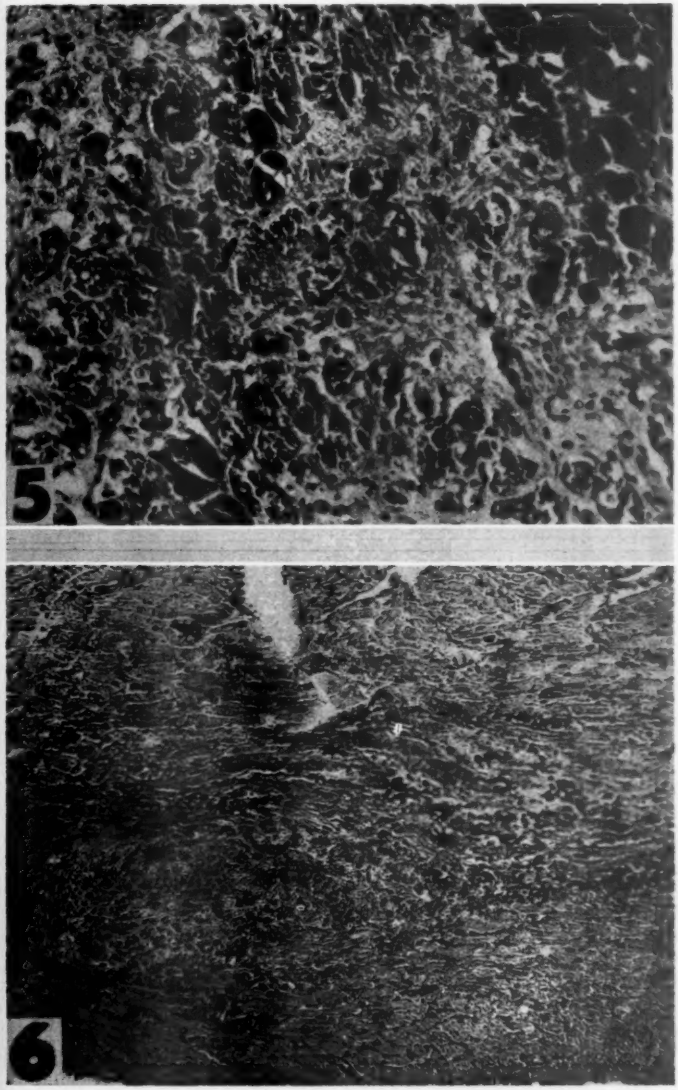

FIG. 5. Myocardium of rat treated with 400 γ/day DCA and subjected to ESS. Note degeneration and necrosis of muscle fibres and their replacement by connective tissue. Gomori's trichrome x 260.

FIG. 6. Granuloma formation in the heart of rate treated with 200 γ/day DCA. Note infiltration and separation of muscle fibres by inflammatory cells. Gomori's trichrome x 130.

TABLE 1

Data		GROUP						
		DCA and ESS and RD†	ESS A*	and RD B	DCA and RD	Controls and RD	DCA	Controls
No. animals	Initial	8	8		8	8	6	6
	Final	5	2	3	7	6	4	5
Body wt. (g.)	Initial	73±3‡	73±5		75±4	72±4	69±5	78±5
	Final	166±19	182±3	206±12	173±6	181±13	184±10	175±9
Adrenals	mg/100 g.	36.5±1.7	36.9±0.2	44.4±2.2	38.1±2.4	33.8±3.2	37.4±1.8	34.5±2.1
Thymus		178±60	242±4	181±29	214±9	206±9	198±15	166±29
Heart		492±35	353±7	455±2	438±2	376±13	484±16	400±11
Kidney		906±43	831±38	870±68	1009±34	908±50	1034±66	937±69
Lesions§	Severity				Distribution			
Heart	0	1	2	3	6	6	2	5
	1	2	0	0	0	0	1	0
	2	1	0	0	1	0	1	0
	3	1	0	0	0	0	0	0
Kidney	0	0	0	0	0	6	0	5
	1	2	2	3	5	0	2	0
	2	2	0	0	2	0	2	0
	3	1	0	0	0	0	0	0

* Survivors divided into A (normal) and B (hypertensive) subgroups.

† DCA = Desoxycorticosterone acetate: ESS = Electroshock stress: RD = Restricted diet.

‡ Mean ± S.E. of mean.

§ The criteria established for the grading of lesions were as follows: Heart: 0 = no visible abnormality; 1 = fibrotic or inflammatory foci detectable, but rare; 2 = visible hyaline degeneration in vessel walls, or perivascular granuloma formation; 3 = extensive vascular and/or myocardial necrosis. Kidney: 0 = normal structure; 1 = occasional tubular casts, interstitial foci of inflammatory cells and/or frank enlargement of parietal layer in Bowman's capsule; 2 = numerous casts, occasional capillary loops obliterated or hyalinized; 3 = extensive hyalinization or necrosis of vessel walls with tubular casts everywhere common.

Whether ESS augmentation of DCA hypertension is to be ascribed to hormonal sensitization of the cardiovascular system to the effects of stress, stress sensitization of the vascular system to the hormonal effects, or to a superimposition of endogenous adrenal secretion upon the administered hormone is as yet uncertain. In addition to the fact that numerous other mechanisms could be evoked, conceivably electric shock may induce a neurogenic hypertension, or impose a neurogenic component upon hormonal hypertension. It has recently been reported that rats with renal hypertension exposed to intermittent psychological stress—which also, incidentally, involved periodic electric shocks although applied less frequently than in the present instance.—suffered greater cardiovascular damage than those subjected to renal injury alone, although the blood pressures exhibited by the respective groups did not differ appreciably (12). By contrast, the present findings indicate that with DCA induced hypertension, under the circumstances described, stress influenced the hypertension to a much greater degree than it did the associated

TABLE 2

Data		DCA and ESS*	DCA	ESS	Control
No. animals	Initial	8	8	8	8
	Final	7	8	3	6
Body Wt. g.	Initial	88±3†	88±3	85±3	85±2
	Final	204±9	215±8	210±11	191±5
Thymus	mg/100 g.	105±6	146±13	124±27	163±6
Adrenals		36.3±1.4	32.5±1.7	34.8±2.1	35.3±3.3
Heart		398±9	355±8	331±8	323±9
Kidney		971±26	887±36	793±33	839±47
Lesions‡	Severity		Distribution		
Heart	0	5	8	3	6
	1	2	0	0	0
	2	0	0	0	0
	3	0	0	0	0
Kidney	0	4	8	3	6
	1	3	0	0	0
	2	0	0	0	0
	3	0	0	0	0

* DCA = Desoxycorticosterone acetate: ESS = Electroshock stress.
† Mean ± S.E. of mean.
‡ Criteria as in table 1.

structure; 1 = occasional tubular casts, interstitial edema; 2 = extensive hyalinization or necrosis of vessel walls with tubular casts everywhere common. occasional capillary loops obliterated or hyalinized; 3 = extensive hyalinization or necrosis of vessel walls with tubular casts everywhere common.

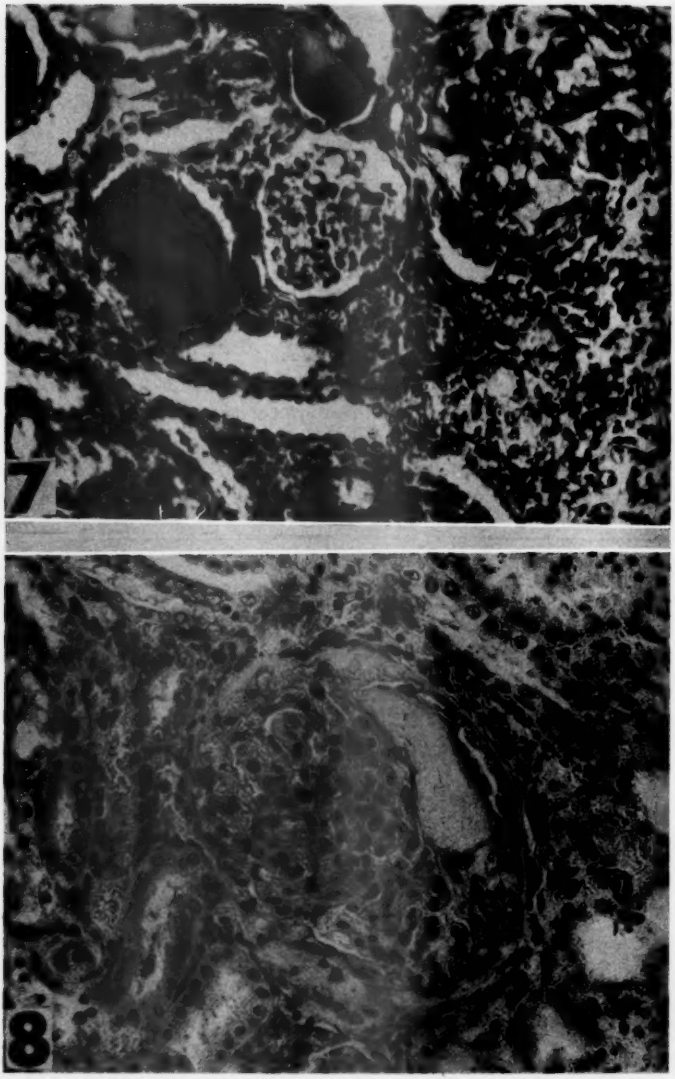

FIG. 7. Kidney from same animal as heart in figure 4. To the upper right of the relatively normal appearing glomerulus in the center, is one that is greatly enlarged and necrotic. Hyaline casts are visible in the tubular lumens. Gomori's trichrome x 260.

FIG. 8. Kidney from animal treated with 200 γ/day DCA. The central glomerulus shows beginning hyalinization, although involvement is less severe than in preceding figure. Hematoxylin and Eosin x 260.

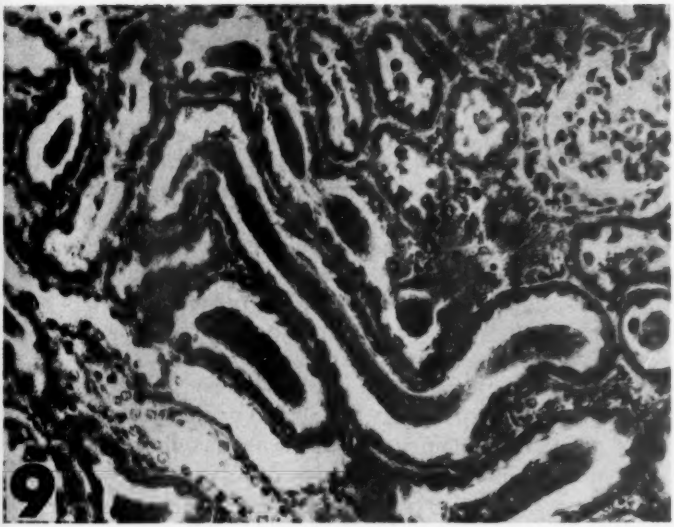

FIG. 9. Kidney from animal exposed to ESS alone. Many hyaline casts are visible in the tubular lumens. The glomerulus in the upper right hand corner appears somewhat shrunken. Gomori's trichrome x 260.

cardiovascular lesions, for whereas the former was clearly influenced by stress in both experiments, only in the second experiment were the lesions prominently affected thereby.

ESS alone while capable of producing hypertension and cardiac hypertrophy, caused only minimal tubular damage in the kidneys, and pathologic changes in the heart were not observed. Uninephrectomized controls given NaCl solution developed neither hypertension nor cardiovascular-renal lesions.

Although the second experiment was continued for longer than the first and at double the hormone dosage, the response of steroid treated animals was not appreciably greater in terms of arterial pressure and was actually less in terms of cardiovascular lesions. It may be that in respect to the induction of hypertensive cardiovascular disease 400γ/day does not appreciably differ from half that amount, also in the second experiment the animals with which the experiment was begun were larger, and the NaCl solution which they consumed was of lower concentration. Both of these factors would independently tend to reduce the response of the animals, and together they might well summate.

Future studies will have to be undertaken in order to ascertain whether the augmentation of pressor response observed in ani-

mals exposed to both ESS and steroid treatment represents a mere summation of their independent effects, or a sensitization to the effects of one by the other.

Summary

Electroshock stress imposed upon rats subjected to uninephrectomy and a high NaCl intake induced hypertension, hypertrophy of the cardiac ventricles and minimal pathologic changes in the kidney. Administered to animals treated with small amounts of DCA, which alone produces hypertension, elevated blood pressure was earlier in onset and reached higher levels. Cardiovascular-renal lesions were most pronounced in animals exposed to both DCA and electroshock stress.

The necessity of depriving animals of food and water for 8 hours a day, six days a week, did not affect their responsiveness to DCA induced hypertensive cardiovascular disease, therefore suggesting that it would not seriously interfere with the response to electroshock stress, insofar as the effects on blood pressure are concerned.

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[b] Desoxycorticosterone acetate was generously supplied by Dr. Robert Gaunt, Ciba Pharmaceutical Corp., Bloomfield, N. J.

STUDIES ON 4-AMINOPYRAZOLO(3, 4-d)PYRIMIDINE: REVERSAL OF CELLULAR DAMAGE IN THE HELA STRAIN[a]

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Studies with various test systems have shown that 4-aminopyrazolo(3, 4-d)pyrimidine (synonyms pyrazoloadenine or 4-APP) is an effective antimetabolite and exerts more or less differential inhibitory action upon growth of malignant and nonmalignant tissues (Skipper *et al.*, 1955; Hsu *et al.*, 1956; Fuerst *et al.*, 1956). Metabolic studies concerning 4-APP have been briefly reported by Feigelson and Davison (1957). Investigations carried out in our laboratories revealed that this compound was quite toxic to several *in vivo* tissues of mice, especially to the liver. Prolonged treatment also caused blindness in these animals. Clinical tests performed at the Sloan-Kettering Institute (Burchenal, 1956) also showed that the agent produced severe hepatic damage.

Usually the antitumor effect of antineoplastic antimetabolites can be somewhat reversed by the supposed normal metabolite with which the antimetabolite is thought to compete. Attempts to relieve the growth inhibition effect of 4-APP have been made with *Neurospora crassa* (Fuerst *et al.*, 1956; Somers *et al.*, 1957). Similar experiments were conducted employing the HeLa strain as the test system for the neutralization power of various agents on cellular damage produced by 4-APP so as to suggest the possible metabolic pathways with which 4-APP interferes. The findings from these experiments are reported in this communication.

Material and Methods

The methods of maintaining the HeLa strain and of setting up cultures for experiments were essentially the same as described in a previous paper (Hsu *et al.*, 1956). Roller tube cultures were set up on 12 x 50 mm. cover slips with plasma clot and 2 ml. of fluid nutrient was added to each tube. After incubation for four days, each agent was tested alone for its toxicity at 4, 2, 1, 0.5, 0.1 and 0.01 milimolar concentrations. The original fluid was replaced by 2 ml. of fresh nutrient containing the test agent. The control series received fresh nutrient alone. A parallel series was set up to detect the relieving power

of the agent. The test fluid in this set of experiments was prepared by introducing the agent in question (again in various concentrations) to a medium containing a 2 milimolar suspension of 4-APP. 2 ml. of the fluid thus prepared was used to replace the original nutrient.

In all cases, treatment lasted for 24 hours. The cultures were fixed after the treated period with methanol and stained with MayGrünwald-Giemsa. Percentage of mitotic figures and percentage of pyknotic nuclei were used as criteria for measuring the degree of cellular damage.

Results and Discussion

The following list contains the names of various agents that failed to give demonstrable relief of cellular damage in the HeLa strain produced by 2 mMol 4-aminopyrazolo(3,4-d)pyrimidine. Most of the purines and pyrimidines themselves were only slightly toxic at high concentrations after 24 hours of treatment. Even unsubstituted purine which produced severe damage in mouse cells (Biesele *et al.*, 1955) did not produce more than a low degree injury in HeLa cells. However, uracil is noticeably toxic and 2,6-diaminopurine is extremely toxic.

Agents that failed to give relief of cellular damage produced by 4-APP:

Pyrimidine
Cytosine
5-methyl cytosine
Thymine
Uracil
Purine
2,6-diaminopurine
Isoguanine-sulphate
Guanosine
Xanthosine
Guanosine-5-phosphate
Isoguanine-monophosphate
Inosine monophosphate
Flavine adenine monophosphate
Thiamin
Pyridoxine
4-aminopyrazolo(4,3-d)-pyrimidine
6-methyl-4-aminopyrazolo(3,4-d)pyrimidine

In practically all cases when the agent failed to relieve the inhibition of mitotic activities, it also failed to relieve the production of pkynosis. In the case of 2,6-diaminopurine, where the agent itself is highly toxic, the addition of 2,6diaminopurine to 4-APP gave additive damage to the HeLa cells.

Tables 1 and 2 summarize the toxicity data of the several agents themselves, that will be referred to in tables 3 and 4. Table 1 shows their effects on mitosis and table 2, on pyknosis.

TABLE 1

Percentage of mitotic figures in the cell populations of the HeLa strain after treatment with various agents. 2 ml. fluid nutrient per culture tube, 24 hours. Each sample is based upon at least 3,000 cells. . ., no data.

Agent	Concentration of agent (milimolar solution)						Medium Control
	0.01	0.1	0.5	1.0	2.0	4.0	
adenine	2.8	3.5	3.1	4.0	4.9	4.3	2.3
guanine	3.1	2.2	. .	2.1	2.3	2.0	2.6
hypoxanthine	5.0	4.7	2.9	3.4	3.5	2.6	3.3
xanthine	. .	2.0	2.0	2.2	1.2	1.1	2.2
adenosine	3.5	4.4	4.6	3.1	3.7	3.5	3.5
adenosine-5-PO4	4.3	3.9	. .	3.5	. .	3.1	3.6

TABLE 2

Percent of pyknotic nuclei in the cell populations of the HeLa strain after treatment with various agents. 2 ml. fluid nutrient per culture tube, 24 hours. Each sample is based upon at least 3,000 cells. . ., no data.

Agent	Concentration of agent (milimolar solution)						Medium Control
	0.01	0.1	0.5	1.0	2.0	4.0	
adenine	2.0	3.4	2.7	3.3	3.7	3.6	1.9
guanine	1.7	2.7	. .	2.4	2.0	9.6	1.5
hypoxanthine	0.7	0.7	4.5	0.7	3.1	2.1	0.5
xanthine	. .	2.2	2.4	2.6	3.1	5.4	2.2
adenosine	1.4	1.5	0.5	1.0	1.0	1.0	0.8
adenosine-5-PO4	0.7	2.0	. .	0.8	. .	1.5	0.5

It is rather obvious from these two tables that most of these agents are relatively nontoxic or only slightly toxic at high concentrations. It should be mentioned here that an increase of the frequency of mitotic figures observed in some instances does not necessarily mean an increase of mitotic activity. An agent or an environmental factor that increases the duration of mitosis may produce a false positive result. In the case of adenine, for example, the increase of mitotic frequency may be the reflection of slight injury suggested by the increase of pyknotic nuclei as shown in table 2.

Agents listed in tables 1 and 2 were individually incorporated in medium containing a 2 milimolar suspension of 4-aminopyrazolo(3,4-d)pyrimidine to estimate their capacity of relieving the cell damage. The results are shown in tables 3(mitosis) and 4(pyknosis). Xanthine should actually be placed at the borderline between the relievers and the nonrelievers. It did not restore the activity of mitosis at all, but there is some suggestion that it

reduced the amount of pyknotic elements at higher doses. Adenine, guanine, adenosine and adenosine-5-phosphate were positive relievers. Not only the mitotic and the pyknotic values approached those of the controls, but also mitosis was represented by all phases, indicating no arrest at any stage. At lower concentrations, such as 0.1 and 0.01 milimolar solutions, however, the frequency of the pyknotic nuclei increased considerably. Even at these concentrations the frequencies of the pyknotic nuclei were not as high as those produced by 4-APP without the relieving agents.

TABLE 3

Percentage of mitotic figures in the cell population of the HeLa strain after treatment with 4-APP plus relieving agents. 2 ml. fluid nutrient per culture tube, 24 hours. Each sample is based upon at least 3,000 cells. . .., no data.

Agent	Concentration of agent (milimolar solution) in 2 milimolar suspension of 4-APP							Medium Control
	4-APP Control	0.01	0.1	0.5	1	2	4	
adenine	0.2	2.7	5.8	4.6	6.1	3.9	3.3	2.3
guanine	0.0	2.0	2.0	2.5	2.1	3.7	5.5	3.2
hypoxanthine	0.5	2.8	3.5	3.1	3.3	2.6	2.7	3.4
hypoxanthine	0.2	2.4	2.4	..	2.4	3.8	2.1	2.5
hypoxanthine	0.0	..	0.1	..	0.2	0.3	0.3	2.8
xanthine	0.1	..	..	0.1	0.1	0.2	0.1	2.8
xanthine	0.1	..	..	..	0.0	0.2	0.5	2.6
adenosine	0.3	1.8	2.6	3.1	2.7	2.7	1.7	3.4
adenosine-5-PO4	0.1	0.8	1.1	3.3	3.9	3.5	4.5	3.2

TABLE 4

Percentage of pyknotic figures in the cell population of the HeLa strain after treatment with 4-APP plus relieving agents. 2 ml. fluid nutrient per culture tube, 24 hours. Each sample is based upon at least 3,000 cells. . .., no data.

Agent	Concentration of agent (milimolar solution) in 2 milimolar suspension of 4-APP							Medium Control
	4-APP Control	0.01	0.1	0.5	1	2	4	
adenine	11.5	6.9	5.5	2.0	2.1	3.1	2.8	1.9
guanine	12.3	8.2	4.4	2.1	2.2	2.0	2.5	2.2
hypoxanthine	12.9	2.3	11.8	4.0	5.1	7.4	24.7	0.7
hypoxanthine	8.5	3.7	2.0	..	2.7	1.8	2.0	0.8
hypoxanthine	12.1	..	9.9	..	11.5	7.4	5.6	3.2
xanthine	11.1	..	..	5.4	5.4	3.5	8.7	1.7
xanthine	18.9	..	..	..	11.7	8.6	6.0	1.2
adenosine	17.7	5.6	2.9	2.9	5.6	7.9	9.5	0.8
adenosine-5-PO4	17.3	11.6	7.6	3.4	1.0	3.7	1.0	0.6

Hypoxanthine gave erratic results in relieving the 4-APP damage. The substance is not highly toxic. In fact, at lower concentrations it showed some mitosis-promoting abilities. When hypoxanthine was used together with 2 mMol 4-APP, it exerted no neutralization power in one set of experiments but showed definite relief in another. In still another test the effect of hyproxanthine was erratic within the set.

Biesele (1954, 1955) found that coenzyme A exerted some neutralizing activity on the damage of sarcoma 180 cells produced by 6-mercaptopurine. It is of interest to note that the same compound showed some activities against 4-APP. Flavine adenine diphosphate, active against 4-APP in Neurospora systems (unpublished data), was a rather strong reliever in the HeLa system. Tables 5 and 6 include preliminary results on these two agents.

If the data on 4-APP relief in the HeLa cultures are compared with those in the Neurospora system, a number of differences can be pointed out immediately. Many compounds that are relievers in the Neurospora system are inactive in the HeLa

TABLE 5

Percentage of mitotic figures in the cell populations of the HeLa strain after treatment with 4-APP plus either CoA or FAD. 2 ml. fluid nutrient per culture tube, 24 hours. Each sample is based upon at least 3,000 cells. . ., no data.

Agent	Concentrations of agent (by weight) in 2 milimolar suspension of 4-APP					Medium Control
	4-APP Control	.00005	.0005	.005	.05	
CoA	0	0.06	0.06	0.6	2.0	2.8
FAD	0.2	. .	0.4	1.0	6.7	3.3

TABLE 6

Percentage of pyknotic figures in the cell populations of the HeLa strain after treatment with 4-APP plus either CoA or FAD. 2 ml. fluid nutrient per culture tube, 24 hours. Each sample is based upon at least 3,000 cells. . ., no data.

Agent	Concentrations of agent (by weight) in 2 milimolar suspension of 4-APP					Medium Control
	4-APP Control	.00005	.0005	.005	.05	
CoA	10.7	10.1	7.8	5.7	4.8	0.8
FAD	14.0	. .	8.9	2.4	1.8	1.6

cultures. These include thiamine, pyridoxine, 6-methyl-4-aminopyrazolo(3,4-d)pyrimidine, 4-aminopyrazolo(4,3-d)pyrimidine, etc. On the contrary, compounds like adenosine-5-phosphate, which was quite efficacious in relieving cellular damage in HeLa, showed an extremely weak effect in Neurospora. Comparing them we note that the two test systems are not only dissimilar in their material, one being a lower fungus and the other, human cancer cells, but also differ in respect to the criteria for toxicity. In Neurospora, the compounds were added to the medium before the growth of the organisms took place. In the HeLa cultures, on the other hand, vigorous growth (presumably the log-phase) was initiated before the agents were introduced. Moreover, in Neurospora, the organisms were incubated with the agents until the time for harvest whereas in HeLa a 24-hour period was arbitrarily set as the period for treatment. This situation might well be different had the HeLa cultures been initiated in the medium containing the agents.

Experimental data on Neurospora led us to postulate the possibility that the compound 4-aminopyrazolo(3,4-d)pyrimidine interferes with DNA synthesis as well as thiamine synthesis. In HeLa, no suggestion of thiamine involvement was indicated. The compounds giving relief of cellular damage in the HeLa strain seem to indicate the possibility that the RNA route was impaired by the antimetabolite. However, our present data do not permit any suggestion on the enzymatic process or processes upon which 4-APP acts, nor can they assist in depicting the scheme for RNA synthesis.

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A COLORIMETRIC REACTION FOR TOLBUTAMIDE[a]

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Tolbutamide, 1-butyl-3-p-tolylsulfonylurea, has been shown to be effective in controlling the hyperglycemia of diabetes mellitus when administered orally (2,3). However, despite the large number of investigations published on various aspects of the substance, no simple chemical test has been developed to estimate the amount of the substance in body fluids or other preparations. We have attempted to develop colored derivatives by reactions which serve as the basis of colorimetric tests for chemically related substances. For example, since tolbutamide is a sulfonylurea derivative, the colorimetric tests used to identify the sulfonamides and to determine their concentration (1), were tried, but failed to produce a color. Similarly, the various tests for toluene, urea, peptides and proteins all proved fruitless, as well as attempts to prepare a dye from the compound, using the traditional techniques found in the literature. It was discovered, however, that an aqueous solution of the sodium salt of tolbutamide reacted with an alcoholic solution of 2-naphthol, upon the addition of an aqueous solution of sodium nitrite and concentrated sulfuric acid. The result was the production of a greenish precipitate which floated to the surface and which could be dissolved in chloroform to yield an orange to red solution, depending upon the concentration of the tolbutamide in the original solution.

Specifically, 5.0 ml. of a solution of tolbutamide was placed in a test tube and the reagents were added in the following order: 1) 0.5 ml. of a 1 per cent solution of 2-naphthol in ethyl alcohol, 2) 1.0 ml. of a 10 per cent solution of sodium nitrite in water, freshly prepared, and 3) 0.2 ml. of 95 per cent (18 molar) sulfuric acid. Within a period of approximately 10 minutes, a dark greenish oily precipitate formed and floated to the top of the liquid. After the reaction, which obviously involved the production of nitrous acid from sodium nitrite and sulfuric acid, had subsided, 10.0 ml. of chloroform was added to the mixture. The solution was now shaken moderately to extract the colored component from the upper, aqueous layer into the lower, chloro-

form layer. The colored component remained stable for a period of at least 30 minutes. As an example of the applicability of the method, consider a series of solutions of tolbutamide ranging in concentration from 0.10 per cent to 1.0 per cent which were allowed to react according to the above procedure. The data, reproducible to about ± 4 per cent, obtained from this series of solutions are shown in table 1. The readings were made, at 490

TABLE 1

Per Cent Transmission and Optical Density for Various Concentrations of Tolbutamide in Water

Concentration of Tolbutamide in g/100 ml.	Percent Transmission	Optical Density
.10	75	.125
.20	56	.252
.30	45	.347
.40	39	.409
.50	31	.509
.80	21	.678
1.0	14	.854

mu – 520 mu wave length, on a Coleman Universal spectrophotometer, within 30 minutes, against a blank prepared in the same way except that distilled water was substituted for the tolbutamide solution. The reaction could not be elicited with some of the common sulfa drugs such as sulfanilamide and sulfathiazole, with carbutamide (2, 3), or with N^1-beta-phenethylformamidinylimino urea hydrochloride (phenethylbiguanide), which has also been found to be an active oral hypoglycemic agent (5).

The method was found to be applicable to blood serum and urine samples to which equivalent amounts of tolbutamide were added. The results, within about ± 6 per cent variation, were in good agreement with those obtained in water solutions of the drug at the same concentrations. Although the main goal of the technique was not to develop a micro method for the determination of tolbutamide in blood and urine, we have extended the procedure to more dilute solutions of tolbutamide such as those that do occur in blood and urine. This is done by increasing the volume of the sample, employing cells with a longer optical path, namely 30 mm. instead of 15 mm. and utilizing a smaller volume of chloroform for extraction to bring the sample within the useful range of the method. For example, by using a 10 ml. sample

and twice as much reagents, employing cells with an optical path twice as long, and using 5 ml. of chloroform for extraction, the useful range of the method was extended to concentrations of tolbutamide of 0.0125 – 0.125 grams per 100 ml. of solution. Normal serum and urine samples to which varying amounts of tolbutamide were added in this range gave results showing a recovery of about 92 per cent and reproducibility within ± 7 per cent, using as a blank serum or urine to which no tolbutamide was added but which otherwise was treated with reagents and chloroform in the same manner as the samples. The cuvettes employed were of rectangular shape, 5 mm. by 30 mm., inside measurements. If smaller cells were available, that is, cells which would require less than 5 ml. of solution and still have a long enough optical path, we believe that the method could be extended to lower concentrations of tolbutamide. In this way, the method can be utilized to determine concentrations of tolbutamide encountered in therapeutic studies.

Summary

A colorimetric method for the estimation of tolbutamide in aqueous solution has been developed. It involves a reaction between tolubtamide and the following reagents, added in a specific order and concentration: 2-naphthol, sodium nitrite and concentrated sulfuric acid. A red color is produced. The method is found to be applicable to the determination of tolbutamide over the range of concentrations extending from the levels encountered in therapeutic studies up to those employed in purely chemical studies, *i.e.* the range of approximately 5 mg. to 1 g. per 100 ml. of solution. Neither carbutamide nor phenethylbiguanide, two other oral hypoglycemic agents, react under these conditions.

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HISTOLOGIC ALTERATIONS IN THALAMIC NUCLEI OF MAN FOLLOWING CORTICAL LESIONS[a]

GLENN V. RUSSELL[b]

Thalamocortical relationships have been investigated by a large number of workers. One of the methods of choice has been the study of the retrograde cell reaction in the thalamus following a lesion to the cerebral cortex, a method discovered by Gudden (1870) and elaborated upon by von Monakow (1882) and by Nissl (1892). Despite extensive use of the method there is a lack of information on the rate and sequence of histologic alterations occurring in the nuclei of the thalamus following a lesion to the cerebral cortex. This is particularly true in man. Gonda and Malamud (1954) have attributed this scantness of data to the generally recognized observation that post-operative deaths tend to occur either within a few days of surgery, at a time when no histologic alteration has yet occurred in the thalamus, or months to years later, at a time when the surgical lesion has become stationary and the histologic alterations in the thalamus have long since ceased. A similar statement might well be made for "naturally" occurring cerebral damage such as that following a cerebral vascular accident or trauma to the head.

Gonda and Malamud (1954) reported on the histologic alterations in the nuclei of the thalamus in one case of self-inflicted lobotomy of 38-days duration, and were thereby led to study 6 additional cases that had survived lobotomy for periods of from two weeks to 8 months. Among their conclusions they suggested that "reversible" changes of axonal reaction characterize the first three weeks following lobotomy. "Irreversible" changes marked by loss of neurons and reactive astrogliosis appear during the second to fourth months.

Their first conclusion (*viz.*, "The changes in the thalamus are similar in self-inflicted and in surgical lobotomies, . . .") suggested and confirm the thought that carefully selected cases of spontaneous cerebral vascular accident, where the lesion is well limited to the cortex and the immediately subjacent white matter, might add to the limited data on this subject.

Materials

The brains of 24 patients who had had a cerebral vascular accident or other cortical injury have been secured and prepared for microscopic study.[c] In each case the lesion was carefully mapped and blocks were taken from the edges of the lesion to confirm the estimate of cortical areas involved by the destruction. The thalamus of each case was blocked and imbedded in celloidin and serially sectioned at 30 to 40 microns. Every tenth section from the thalamus and selected sections from the cortical blocks were stained with cresyl echt violet for cells.

Upon careful study of the series thus obtained, 11 cases were discarded because of (1) a lesion which was too extensive and penetrated deeply into the white matter of the hemisphere, (2) incidental and unsuspected thalamic lesions, and/or (3) evidence of extensive generalized arteriosclerotic disease. Any one of these would have invalidated the observations of histologic change or neuron loss in the nuclei of the thalamus. The remaining 13 cases were adjudged usable for this study. Assessment of neuron loss was made by actual counts of the numbers of neurons in the affected nucleus and comparison with the opposite unaffected nucleus.

Observations

Of the 13 usable cases 7 had had the cortical lesion for a period of 2½ months or more prior to death. Four cases had lesions which were from 2–3 days to 18 days of age at the time of death. Two cases died 25 and 58 days after neurosurgery (Grantham lobotomy) and are included in this series. Thus there are 7 "old" and 6 "recent" cases in which the histologic alterations in the thalamus following cortical lesion can be assessed.

In the 6 "recent" cases the cortical lesion varied in age from 2–3 days to 58 days. Although it is not the purpose of this communication to discuss in detail the specific relationship between a given cortical area and a specific thalamic nucleus, nonetheless it is necessary to describe briefly the site of the cortical lesion and the region of the thalamus studied for histologic change.

Case T-9641: This brain had a recent hemorrhagic softening in the frontal cortex and subjacent white matter in the area of the projection of the medial dorsal nucleus, and a second area in the white matter immediately subjacent to the area of the cortical projection of the pulvinar. These two areas were estimated to be 2–3 days old. An older cortical softening, estimated to be 14 days old according to the history, was found in the right middle frontal and superior frontal cortex. Examination of the medial dorsal nucleus and the pulvinar revealed no descernible

histologic alteration in either the neurons or the glial elements. The ventral lateral nucleus, which projects to the area of the older cortical lesion, showed a minimal diffuse axonal reaction in approximately one-half of its neurons. There was no glial reaction.

Case T-9678: This brain showed a cortical softening which involved the inferior one-third of the Rolandic sensory-motor cortex. From the history this lesion was 6 days old. Examination of the ventral lateral and the ventral postero-medial nuclei which project to these cortical areas revealed no discernible histologic alterations in either the neuronal or the glial elements.

Case T-9646: This brain had an extensive cortical softening which involved the bulk of the distribution of the right middle cerebral artery. Swelling of the brain as a result of this lesion had caused herniation of the anterior and middle portions of the gyrus cinguli beneath the falx and softened this cortex. From this history it was evident that the initial insult had occurred 15 days prior to death. Comparison of the two areas of malacia suggested that the cingulate lesion was only slightly younger than the primary lesion. Examination of the thalamus revealed a diffuse axonal reaction involving all of the neurons of the anterior nucleus, the nucleus which projects to the cingulate cortex destroyed by the herniation. Comparison with the unaffected anterior nucleus gave a suggestion of slight loss of neurons in the affected nucleus. A somewhat more acute axonal reaction was noted in the neurons of the ventral group of nuclei, particularly in the ventral lateral, ventral postero-medial, and the medial zone of the ventral postero-lateral nuclei. Comparison with the unaffected side showed a 20 per cent reduction in the numbers of neurons in these nuclei. There was a suggestion of some satellitosis about the acutely involved neurons, but no clear glial reaction was as yet present.

Case T-9989: This brain showed a large lesion in the cortical distribution of the left middle cerebral artery. From the history it was evident that this lesion was 18 days old. Examination of the ventral group of nuclei revealed an acute axonal reaction in most of the neurons of the ventral lateral, ventral postero-medial, and the medial zone of the ventral postero-lateral nuclei. There was a 25–30 per cent reduction in the numbers of neurons in

these nuclei as compared with the unaffected side. Minimal astrocytic gliosis and moderate satellitosis was noted.

Case T-9939: A Grantham lobotomy procedure had been carried out 25 days prior to death. This procedure is designed to interrupt the radiations from the medial dorsal nucleus to the frontal cortex. Examination of the medial dorsal nucleus revealed a severe axonal reaction in all of the cells of this nucleus which project to the cortex. There was a 25–30 per cent loss of neurons in these nuclei as compared with a large number of counts of the normal cell density of this nucleus. A moderate astrocytic gliosis was evident in the affected nuclei.

Case T-9911: This brain had also had a Grantham procedure performed bilaterally 58 days prior to death. Examination of the medial dorsal nuclei revealed a 50–60 per cent loss of neurons with a severe axonal reaction in all of the residual neurons. Moderate astrocytic gliosis with marked satellitosis was present throughout the nuclei.

The 7 "old" cases in this series were observed to have cortical lesions that were older than 2 to 2½ months. A brief description of the cortical involvement and the thalamic changes follows.

Case T-9582: The cortical lesion in this case (reported in detail elsewhere; Russell, 1954) was 7 years old and involved the area of distribution of the anterior nucleus, the medial dorsal nucleus, and parts of the projections of the ventral nuclei. The anterior and medial dorsal nuclei showed complete loss of neurons and the region of these nuclei was reduced to a dense fibrous glial scar. The ventral nuclei showed areas of complete neuron loss compatible with the magnitude of the cortical injury. Gliosis was evident in the damaged parts of the ventral nuclei.

Case T-29662: This brain had complete cortical destruction of the inferior one-third of the right Rolandic sensory-motor cortex as the result of a head injury 7 years prior to death. The ventral postero-medial nucleus and the inferior and medial parts of the ventral postero-lateral nucleus on the right showed complete loss of neurons and the area of these nuclei had been reduced to a dense fibrous glial scar.

Case T-9665: This brain had an area of cortical loss which included the middle one-third of the precentral gyrus on the right side. According to the history this lesion was 7 months of age. The ventral postero-lateral nucleus of the thalamus on the right

side contained a zone adjacent to the ventral postero-medial nucleus that was devoid of neurons. The gliosis, while severe, did ont appear to be as densely fibrous as in the preceding two cases.

Case T-9668: This brain showed several areas of old cortical damage which, from the history, were all over two years of age. The best delimited area involved the middle one-third of the right pre- and post-central gyri. In the right thalamus there was a zone of complete loss of neurons which included the medial one-half of the ventral postero-lateral nucleus and which extended rostrally into the medial part of the ventral lateral nucleus. This region had been reduced to a dense fibrous glial scar.

Case T-9687: This brain showed an area of cortical damage that included all of the medical aspect of the left occipital lobe. From the history it was apparent that this lesion was 1 year old. The entire inferior pulvinar on the left side was devoid of neurons and had been reduced to a dense fibrous glial scar. The lateral geniculate body on the same side was completely degenerated, however, the gliosis in this nucleus was much less severe than that in the adjacent inferior pulvinar.

Case T-9691: In this brain the cortex of the posterior part of the superior frontal gyrus and the anterior part of the paracentral lobule on the left was desroyed. No history was available, however, from the appearance of the lesion it was quite old. Examination of the thalamus revealed that the lateral one-third of the ventral lateral nucleus adjacent to the nucleus reticularis was completely devoid of neurons and had been replaced by a dense fibrous glial scar.

Case T-10067: In this brain there was a cortical lesion which involved the posterior one-half of the angular gyrus and the caudally adjacent superior occipital gyrus. Although no detailed history was available it was clear from the appearance of the lesion that it was quite old. Examination of the thalamus revealed that the lateral and ventral portion of the pulvinar was devoid of neurons and had been reduced to a dense fibrous glial scar. The inferior pulvinar was not affected.

Discussion

The observations upon the 6 brains with "recent" lesions in this series, together with the 4 cases reported by Gonda and

Malamud (1954) and the one case reported by Powell (1952), are summarized in table 1. They have been arranged in chronological order based upon the age of the lesion. The 7 "old" cases and the 3 "old" cases reported by Gonda and Malamud (1954) are not listed separately, but are grouped together at the bottom of the table.

It is evident that the histological alterations which occur in the nuclei of the thalamus after cortical lesion form an orderly sequence of events. Although there appears to be some variability

TABLE 1

Case*	Age of Lesion	Thalamic Nucleus Examined	Neuronal Alterations	Glial Alterations
T–9641	2–3 d	Medial dorsal and Pulvinar	No alterations	No alterations
T–9678	6 d	Ventral nuclei	No alterations	No alterations
T–9641	14 d	Ventral lateral	Minimal diffuse axonal reaction	No alterations
G&M	14 d	Medial dorsal	Diffuse axonal reaction	Suggestion of increase in oligodendroglia
T–9646	15 d	Anterior nucleus	Diffuse axonal reaction with slight cell loss	No alterations
		Ventral nuclei	Acute axonal reaction with 20% cell loss	Suggestion of slight satellitosis
T–9989	18 d	Ventral nuclei	Acute axonal reaction with 25–30% cell loss	Minimal astrocytic gliosis with moderate satellitosis
G&M	21 d	Medial dorsal	Acute axonal reaction with minimal cell loss	Marked satellitosis
P	24 d	Anterior nucleus, LG, MG, Pulvinar	Complete cell loss	Severe astrocytic gliosis
		Ventral nuclei	Acute axonal reaction with 50% cell loss	Moderate astrocytic gliosis
T–9939	25 d	Medial dorsal	Severe axonal reaction with 25–30% cell loss	Moderate astrocytic gliosis
G&M	38 d	Medial dorsal	Severe axonal reaction with 50% cell loss	Slight astrocytic gliosis
G&M	42 d	Medial dorsal	Severe axonal reaction with 50% cell loss	Slight astrocytic gliosis
T–9911	58 d	Medial dorsal	Severe axonal reaction with 50–60% cell loss	Moderate astrocytic gliosis
2½ months or more of age.		.....	Essentially complete loss of neurons	Astrocytic gliosis, first cellular, then fibrous.

* Numbered cases are those described in this communication. Those designated "G&M" are from the communication of Gonda and Malamud (1954). That one marked "P" is from the communication of Powell (1952). The cases with lesions 2½ months or older are grouped together and include 3 reported by Gonda and Malamud and the 7 from this group.

between individual cases, an occurrence which was not unexpected, and some variability in the rate of change between different nuclei of the thalamus in a given case, a fact previously alluded to by Walker (1938), the general pattern is quite clear and confirms the suggestions previously made by Gonda and Malamud (1954). The sequence of histologic alterations that occur lend themselves to a separation into three periods, or Phases, and these are represented schematically in figure 1.

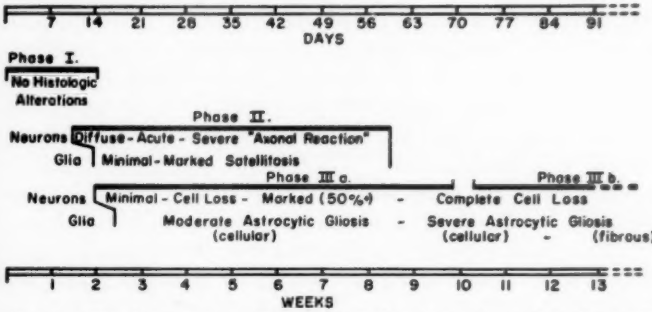
7 14 21 28 35 42 49 56 63 70 77 84 91
DAYS
Phase I.
No Histologic Alterations
Phase II.
Neurons Diffuse - Acute - Severe "Axonal Reaction"
Glia Minimal - Marked Satellitosis
Phase III a.
Phase III b.
Neurons Minimal - Cell Loss - Marked (50%+) - Complete Cell Loss
Glia Moderate Astrocytic Gliosis (cellular) - Severe Astrocytic Gliosis (cellular) - (fibrous)
1 2 3 4 5 6 7 8 9 10 11 12 13
WEEKS

FIG. 1. The three Phases of histological alterations observed in the nuclei of the thalamus of man following a lesion of the cerebral cortex.

Phase I is the period during which no histologic alteration is observed either in the neural or the glial elements of the thalamus subsequent to destruction of the cerebral cortex. The Phase appears to last for about two weeks.

Phase II is the period of "reversible" alteration during which the classical axonal reaction is observed in the neurons of those thalamic nuclei which project to the cortical region that has been damaged. The glial alteration that is associated with this Phase is the apparent increase in the numbers of oligodendroglia and the arrangement of these cells as satellite glia about the reacting neuron.

The early minimal reactions of a retrograde nature are difficult to assess in human autopsy material, however, it is clear that Phase II begins towards the end of the second week subsequent to the cortical injury and that the first evidence of this in the thalamus is a minimal axonal reaction in some of the cells of the affected thalamic nucleus. Some minimal satellitosis may or may not be evident at this time. The severity of the axonal reaction

may be slightly greater in the ventral nuclei in the early period of Phase II, but progresses rapidly in all nuclei during the third and fourth weeks subsequent to cortical injury. As the axonal reaction progresses neurons are lost (Phase III), but as late as the end of the second month subsequent to the cortical insult some residual neurons showing a moderate to severe axonal reactio may still be found. It is, however, apparent that all thalamic neurons which show a well defined avonal reaction after cortical damage will eventually be lost. The glial alterations during the later stages of Phase II would be those of increasing satellitosis and perhaps the beginnings of astrocytic proliferation.

Phase III is the period of "irreversible" alteration including the actual loss of neurons and the progression of astrocytic gliosis. This phase begins with the first drop-out of neurons during the third week. The rate at which neurons are lost, although steadily progressive, is the most variable feature of the histologic alterations studied. This may be due to either, or both, of two causes: (1) differences in the actual extent of the cortical damage among different cases; and (2) differences in the detailed morphology of individual neurons and neuron groups (nuclei) of the thalamus. From the present study it is not possible to assign a predominant role to either of these two possibilities, although I would prefer the latter.

Marked loss of neurons (50 per cent or more) is evident in those thalamic nuclei that project to a destroyed cortical area during the second month subsequent to the cortical injury. This loss appears to have been completed early in the third month, although additional data are required for the period from 60 to 90 days subsequent to cortical injury. Shortly after neurons begin to drop-out a cellular astrocytic gliosis develops. This gliosis is at first slight to moderate in degree, but by the third month has become rather severe. With the passage of time the gliosis alters its appearance from the early cellular picture to the well defined dense fibrous glial scar.

On the basis of neuronal and glial alterations, Phase III is separated into Phase IIIa, the period of neuron drop-out and moderate cellular astrocytic gliosis, and Phase IIIb, the period of complete neuron-loss and severe astrogliotic change. Phase IIIa is a transient period lasting perhaps two months, while Phase

IIIb is the permanent and final picture seen in the thalamus, barring further damage to the cerebral cortex.

No real overlap between Phase I and Phase II can occur, for there is or there is not observable histologic alteration in a given thalamic nucleus. The overlap between Phase II and Phase III occurs by definition. The former period deals with the axonal reaction and the glial satellitosis. The latter emphasizes the loss of neurons and the development of the permanent gliotic scar. This overlap should focus attention on the very noticeable differences in the time of onset of the axonal reaction, the rate at which this reaction progresses, and the time that it takes for particular thalamic neurons to be finally lost. These differences exist between neurons within a given thalamic nucleus, and between neurons in different thalamic nuclei. An explanation for these differences is not at present available.

Powell (1952) reported the occurrence of residual neurons, most of them showing an acute axonal reaction, in a case 24 days after virtual hemidecortication (surgical). At the time he was unable to explain the phenomenon. Later I also noted this (1954) and was equally at a loss to explain it. This report does offer a partial solution, for it is now evident that insufficient time had elapsed subsequent to the cortical injury for complete drop-out of thalamic neurons to have occurred.

Conclusions

The histologic alterations that occur in thalamic nuclei following cortical lesions in man have been reviewed in 13 cases studied by the author and comparisons made with 8 cases previously reported in the literature. An attempt is made to establish the rate and sequence of neuronal and glial changes with the passage of time subsequent to cortical loss or damage.

1. A period of about two weeks elapses subsequent to cortical insult during which no histologic alteration is observable. This period is designated Phase I.

2. From the middle of the second week after cortical insult, first a few and then increasing numbers of thalamic neurons show a progressively more severe axonal reaction. This is accompanied by an increasing satellitosis. This period is designated Phase II and may last well into the second month subsequent to the time of the cortical insult.

3. Beginning shortly after the development of the axonal reaction there begins a progressive loss of neurons in affected thalamic nuclei, accompanied by a progressive astrocytic gliosis. This period is designated Phase IIIa. Sometime during the third month after cortical insult, loss of neurons is complete and the gliosis, which was at first cellular, becomes progressively more dense and fibrous in character. This period, that of the final picture, is designated Phase IIIb.

4. Although individual differences may well be of importance in the determination of the time of onset and the rate at which thalamic neurons show the axonal reaction and are then lost in different cases, it is strongly suggested that there are important differences in the detailed morphology of individual neurons and neuron-groups (nuclei) of the thalamus.

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ANTITUMOR ACTIVITY OF SOME AMINO ACID ANALOGUES

I. S-CARBAMYLCYSTEINE AND O-CARBAZYLSERINE

CHARLES G. SKINNER, GEORGE F. MCKENNA, TOMMY J. MCCORD, AND WILLIAM SHIVE[a]

Various antimetabolites have been examined as potential antitumor agents; however, the number of compounds which possess significant activity is less than might have been anticipated when this approach was instigated. Among other reasons for this lack of activity is that a strictly competitive amino acid antagonists of moderate activity would be ineffective because of the inability to maintain it at high blood levels necessary for inhibition of the natural amino acids at their normal concentrations. Only noncompetitive or highly effective competitive antagonists of amino acids could exert appreciable effects upon tumors since the concentration of the amino acid in the host would otherwise reverse the inhibition.

Several amino acid analogues have been observed to possess antitumor activity; for example, ethionine (an analogue of methionine) inhibits rat sarcoma (Dunn and Murphy) and augments the effect of a folic acid antagonist on a mouse mammary sarcoma (Shapiro and Fugmann). 3-Thiophenealanine (a phenylalaine antagonist) depressed the growth of sarcoma transplants in rats, and its effect was reversed by the natural amino acid (Wissler *et al.*). Azaserine (O-diazoacetyl-L-serine) (Stock *et al.*) and 6-diazo-5-oxo-L-norleucine (DON) (Ehrlich *et al.*) are reported to inhibit effectively the growth of mouse sarcoma 180. Recently the inhibitory properties of these substances have been shown to involve an inactivation of the enzyme effecting the conversion of formylglycinamide ribotide to formylglycinamidine ribotide. Glutamine has been found to delay, competitively, the inactivation of the enzyme by azaserine (Levenberg, Melnick and Buchanan). Several new glutamine analogues have been synthesized and studied in various biological systems in these

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laboratories, and the present report deals with a study of some of these compounds with respect to their antitumor activity.

Experimental Procedures

Mice of the dba strain, bearing transplanted parts of RC mammary adenocarcinoma were used in all these tests except as noted. The tumors were implanted by subdermal injection in the right inguinal area of 0.02 ml. of tumor tissue dispersed in 0.2 ml. of 0.85 per cent saline solution three days before the test material was introduced. All of the subsequent injections of the test solutions were given by subdermal injection in the left inguinal area; control animals received 0.4 ml. of sterile distilled water concurrently. The schedule of injections was varied according to the particular experiment under study and all animals were usually sacrificed, and the tumors removed and studied, ten days after the initial injection. Effectiveness of antimetabolites was evaluated on the basis of the difference in the average weight of the tumor in the experimental groups as compared with the tumor growth in the control animals as indicated in table 1. The effect of these chemicals on

TABLE 1

Antitumor Activity of Some Glutamine Analogues on Mice Implanted with RC Mammary Adenocarcinoma

Treatment	Number of Animals Used	Dosage, mg.	Time of Experiment, Days	Number of Injections	Biological Response (Control = 100)		Remarks
					Tumor Growth	Body Weight	
S-Carbamyl-L-cysteine	6	2	10	7	70	99	
	6	2	11	9	50	96	
	9	3	10	9	38	87	
O-Carbazyl-DL-serine	9	2	10	2	100	94	
	9	2	10	4	70	97	
	18	2	10	7	28	94	*, †
	9	4	10	1	105	119	
	6	10	10	6	0	75	*
γ-Glutamyl-hydrazine	9	2	10	7	89	57	*
	9	10	10	7	79	95	*

* Extensive liver damage, and spleen was smaller than normal.
† Average of two experiments.

the change in body weight of the animals was also observed. Gross inspection of the viscera was accomplished in most instances; observed changes are noted in table 1. Microscopic sections were not made of the internal organs or tumors. The method described above is similar to that previously reported by Taylor *et al.*, and the RC mammary adenocarcinoma has been used for screening programs in other laboratories (Tarnowski and Stock).

An alternate experimental technique was also used with the RC mammary adenocarcinoma implants in which the tumors were allowed to grow until

they attained a size of approximately 0.5–1.0 cm.^{2}, after which the test materials were administered by subdermal injections as described above. These "treated" animals were compared with control animals held under identical conditions except that they were injected with sterile distilled water instead of antimetabolite. Nine animals were used in assays involving both O-carbazylserine and γ-glutamylhydrazine which were administered for ten days at a level of 10 mg./animal/day. No apparent effects on the rate of growth, relative to the controls were observed in these rapidly growing tumors. The antitumor action of S-carbamylcysteine was studied as follows: Implanted tumors were allowed to reach a size of approximately 0.25 cm.^{2} prior to starting the treatment, which consisted of 7 daily injections of either 4 mg., 6 mg., or 8 mg. per animal of freshly prepared solution of S-carbamylcysteine. After 7 days the animals were examined for change in body weight, tumor size, and then autopsied to determine the extent of visual internal damage. Also studied was the effect of injection of the amino acid analogues upon C_3H mice bred in these laboratories for spontaneous mammary carcinoma. Mice which posssessed tumors of approximately 1 cm.^{2} area for both the control and test animals were selected for study, and the test solutions were injected subdermally at a site distant from the tumors. The growth of the tumors was measured and compared with nontreated control animals under identical conditions at frequent intervals.

The test compounds described in this study were dissolved in sterile distilled water at the appropriate concentration so that 0.4 ml. of the test solution contained the desired dosage. Sufficient γ-glutamylhydrazine solution was prepared to complete an entire test at one time, and kept under refrigeration between treatments. In contrast, both O-carbazylserine and S-carbamyl-cysterine were dissolved in sterile distilled water just prior to injection since they are known to be unstable in aqueous solutions.

Results

The antitumor activities of the glutamine analogues studied in this paper, S-carbamylcysteine (Ravel *et al.*), O-carbazylserine (McCord *et al.*) and γ-glutamylhydrazine (McIlwain *et al.*), were examined in three different types of assay systems. When they were injected into C_3H mice bearing rapidly growing spontaneous tumors (mammary carcinoma) essentially no retardation was observed in the rate of growth.

In view of the difficulty frequently encountered in affecting growth of a spontaneous tumor, the major portion of the study was carried out using animals which had been implanted with RC mammary adenocarcinoma cells two or three days prior to injecting the test solutions. Of the three glutamine analogues studied by this technique, S-carbamylcysteine appears to present the most promise as an antitumor agent as indicated by

its overall biological activity in table 1. Treatment of the test animals with either O-carbazylserine or γ-glutamylhydrazine, at a concentration level sufficient to affect the implanted tumor, caused slight, moderate or marked damage to the liver and spleen as observed by autopsy at the conclusion of the experiments. The most probable explanation for the toxicity of the latter two compounds is a hydrolytic cleavage of the molecules *in vivo* to yield hydrazine, which would not be formed from S-carbamylcysteine. S-carbamylcysteine (at a concetration of 3 mg./animal/day) caused some loss of body weight (87 per cent of the control animals); however, a rather significant decrease in the rate of growth of the tumor was observed (38 per cent of the control animals). When the dosage was decreased to 2 mg./animal/day, no appreciable loss in weight of the animals was observed, and the tumor growth was decreased to between 50 and 70 per cent of the control animals. Injection at a level of 20 mg./day of S-carbamylcysteine is lethal to the animals.

The alternate experimental technique in which the implanted tumors were allowed to grow for about 7 days prior to starting the test treatments gave essentially a negative response with O-carbazylserine and γ-glutamylhydrazine; however, with S-carbamylcysteine the results were more encouraging. Injection of S-carbamylcysteine at a level of 4, 6, or 8 mg./animal/day for 7 days resulted in amount of tumor growth which was 84, 26, and 0 per cent, respectively, of that which occurred in the control animals. The weight of the animals relative to the controls were 87, 89, and 80 per cent, respectively. The 8 mg. dosage produced a total regression of the implanted tumor which was visible and measurable at the beginning of the treatment. On autopsy, the animals receiving the 4 mg. treatment showed no visual evidence of internal damage, the 6 mg. treatment resulted in animals having appreciably smaller spleens than the control animals, and the 8 mg. treatment resulted in both liver and spleen damage to the test animals.

Discussion

The high concentrations of amino acids normally present in the host prevents many competitive antagonists from being effective chemotherapeutic agents. This difficulty can be over-

come with an antagonist which possesses an appreciable affinity for the essential enzymatic site of binding of the corresponding natural amino acid. A non-competitive inhibitory action of the analogue is even more desirable. A chemically reactive center, such as the diazoacetyl group in azaserine or 6-diazo-5-oxonorleucine, could permit an analogue to react irreversibly with an enzymatic site after forming the enzyme-analogue complex, which is in contrast to the reversible natural metabolite-enzyme complex. The introduction of such reactive groups in a molecule could thus afford a mechanism for specific enzyme inactivation by appropriate antimetabolites in living organisms.

The introduction of a more reactive thio ester group in place of a related, but less reactive, ester group of an analogue has been attempted with the amino acid analogue, O-carbamylserine. Growth inhibition caused by the oxygen analogue is reversed competitively by glutamine in *Streptococcus lactis* and *Lactobacillus arabinosus* (Skinner *et al.*). The corresponding glutamine analogue containing a reactive thio ester linkage, rather than the normal ester group, resulted in an analogue, S-carbamylcysteine, which appears to inhibit the same biochemical transformations as does O-carbamylserine, but the corresponding microbiological inhibitions are not reversed by the natural amino acid. In this respect, S-carbamylcysteine resembles the biological activity of azaserine (Ravel *et al.*). O-Carbamylserine is much less effective as an antitumor agent than is the sulfur analogue. It seems possible that this difference in antitumor activity of S-carbamylcysteine and O-carbamylserine results from the ability of glutamine in the host to reverse the inhibitory effects of the oxygen analogue, in contrast to its relative inability to reverse the toxicity of the sulfur analogue. This difference in reversibility by glutamine could be due to the more reactive thio ester grouping exerting some irreversible chemical interaction with an enzyme site(s), or it may be due to S-carbamylcysteine exerting an enhanced affinity for the appropriate binding site(s).

The rather extensive liver damage observed in the case of the hydrazine derivatives, O-carbazylserine and γ-glutamylhydrazine, is not unexpected since these analogues probably yield hydrazine *in vivo*, which is a toxic agent. Since O-carbazylserine shows promise as an antitumor agent, with regard to retarded tumor growth as indicated in table 1, several chemical modifica-

tions of this structure are presently under study in an effort to eliminate this difficulty with liver damage. If the side effects caused by degradation products can be controlled, this analogue offers the basis for an extension of this study to other glutamine analogues of this type as potential antitumor agents.

S-Carbamylcysteine, at high dosage levels, produces a toxic effect on both the liver and spleen similar to that of the analogs which contain a hydrazine moiety. It is possible that this effect may result from specific inhibitions of glutamine; however, S-carbamylcysteine at high concentrations non-competitively inhibits certain enzyme systems unrelated to the functions of glutamine (Ravel), and such an action may be involved in this case.

Summary

Of the three glutamine analogues studied, S-carbamylcystine, O-carbazylserine and γ-glutamylhydrazine, the one which shows the most promise as an antitumor agent is S-carbamylcysteine. The two other derivatives caused damage to the spleen and liver when used at a concentration sufficient to affect implanted RC mammary adenocarcinoma tissue in mice. In contrast, treatment of the test animals with an appropriate level of S-carbamylcysteine caused a significant decrease in rate of growth of the tumor with no evidence of gross internal damage and with no appreciable loss in body weight; however, at high dosage levels it does cause damage to the liver and spleen. At even higher levels it is lethal.

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NOTES ON A TOXIN FROM *GYMNODINIUM BREVE*

THEODORE J. STARR[a]

Catastrophic fish mortalities associated with algal blooms have occurred at various times throughout the world (Connell and Cross, 1950; Ballantine and Abbott, 1957). In the Gulf of Mexico, blooms of *Gymnodinium breve* (Davis, 1948) accompanied massive fish kills which were reported along the west coast of Florida (Gunter *et al.*, 1948), the southeast coast of Texas, and the northeast coast of Mexico (Wilson and Ray, 1956). *G. breve* appears to be widely but sparsely distributed in the Gulf of Mexico. A critical combination of chemical and physical factors sets off blooms (Ketchum and Keen, 1948; Gunter *et al.*, 1948; Hela, 1955; Chew, 1955).

Until recently, studies of the metabolism, physiology, and toxin of *G. breve* were hampered because of the fragile nature and fastidious growth requirements of this organism. Wilson and Collier (1955) developed an enriched sea-water medium which supported growth and serial transfer of unialgal cultures of *G. breve*. Their mass cultures killed fish. Ray and Wilson (1957) then isolated *G. breve* and showed that pure cultures killed fish. They reconfirmed the toxicity of unialgal cultures.

This study describes some procedures for the bioassay of the toxin in unialgal cultures of *G. breve* and some properties of crude toxin preparations.

Materials and Methods

Growth of mass cultures. Unialgal mass cultures of *G. breve* were grown in 20 liter carboys containing 15 liters of the sea-water medium described by Wilson and Collier (1955) with (a) half the amount of soil extract, (b) 3 x the final concentration of $Na_2S \cdot 9H_2O$, and (c) omission of EDTA. Good growth (1 to 3 million living cells per liter) was obtained in 4 to 8 weeks; toxicity experiments were then conducted.

Bioassay organisms. In early experiments the guppy, *Lebistes reticulatus*, was used to compare the potencies of different preparations of *G. breve*. The guppies, bred in quantity in laboratory tanks, were available throughout the year. Although the guppies did not breed in waters of high salinity (approximately 2.8 to 3.3 per cent) they survived at these salinities and could be transferred from fresh water without adverse effects. This ability to withstand high

salinities was a prerequisite for its use in bioassays since most of the tests were made in the sea-water medium (salinity, 2.8 to 3.3 per cent, pH 7.9 to 8.2) described above.

In later experiments mullet, *Mugel cephalus*, were used. Small mullet (approximately 2.8 to 3.0 cm.) were readily available in the immediate vicinity of December through June. As they could not be maintained under our laboratory conditions for more than a week without loss of vitality, they were caught with a dip net when needed (several hundred at a time from one school) and used within 1 to 3 days. Small mullet proved sensitive to changes in salinity. Those caught in low-salinity waters (0.3 to 0.8 per cent) were gradually adjusted to higher salinities (2.8 to 3.3 per cent) by the dropwise addition of saturated NaCl to their holding tanks (100 liters) over a period of 24 to 36 hours. Usually this adjustment was unnecessary.

Responses of bioassay organisms to the toxin. Most of our toxin preparations from unialgal mass cultures (1.5 million cells per litter) killed mullet within 2 to 4 minutes. As the preparations were diluted, the symptoms of distress and time to death were prolonged. Mullet showed the following: within 30 seconds a violent twisting and turning accompanied by cork-screw type movements; then contractions and tail curvature at intervals of 10 to 20 seconds; within 1 to 2 minutes equilibrium is lost and fish may turn upside-down or on its side; opercular movements are irregular and slow; little response to probing and they may remain quiescent; immediately before death a violent burst of activity occurs and the fish dies with mouth and opercula opened. Once equilibrium is lost, removal to non-toxic water does not aid recovery.

Toxin preparations which kill mullet in 2 to 4 minutes kill guppies in 8 to 15 minutes. The response of the guppy is sluggish compared to the mullet but the symptoms are similar. It remains to be seen if the toxin acts alike in both fish.

These symptoms are remarkably similar to those described by Abbott and Ballantine (1957) for *Gobius flavescens* (goby) in a toxic preparation of *Gymnodinium veneficum* (Ballantine, 1956).

Bioassay techniques using mullet. One liter aliquots of a mass culture (final pH 8.15, salinity 3.3 per cent, 7 weeks old) having a living cell count of 1.5 million per liter were frozen overnight in flasks. The contents were thawed in a water bath at room temperature (25±2° C.) and filtered through an AA millipore filter. Dilutions were made from the cell-free preparation with sea-water medium (pH 7.9, salinity 3.3 per cent) to give the following final concentrations: (ml. *G. breve* preparation per 100 ml. final solution) 0.0, 2.5, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0, 75.0 and 100.0. One hundred ml. of each dilution were put into 250 ml. beakers. Mullet were washed in the uninoculated sea-water medium for 15 to 30 minutes. At zero time, fish were added to each dilution and death times recorded.

Results

Preliminary experiments with guppies and mullet indicated that living cultures of *G. breve* were less toxic than killed ones.

In many instances, our test organisms remained active for approximately one hour in a culture of live *G. breve*. However, aliquots of the culture, previously treated to destroy *G. breve*, killed both assay organisms in a matter of minutes. Gentle heating, quick freezing, pH changes, vacuum filtration, changes in osmotic pressure, and addition of certain heavy metals to aliquots of the culture, all increased toxicity.

Standard bioassay death curve for mullet. The standard bioassay death curve (Fig. 1) was made with a cell-free preparation. It represents a composite of 4 experiments with a total of 12 to 16 fish for each dilution tested.

Minimum and maximum death times are indicated by the extremes of the vertical lines. The points at which the assay curve passes through the vertical lines are the average death times of mullet in the indicated concentration of the *G. breve* preparation. As shown, undiluted cell-free preparations (100 per cent) killed mullet within 2 to 4 minutes; with increasing

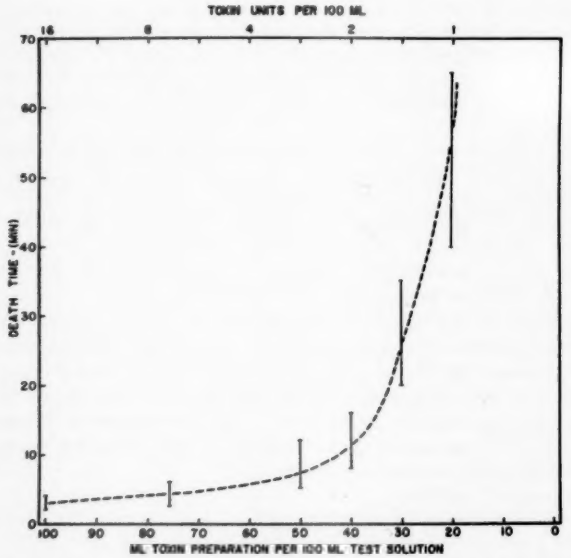

FIG. 1. Response of mullet to *Gymnodinium breve* toxin dilutions. Arbitrary toxin units (TU) defined in text.

dilutions, average death times were increased. In a 20 per cent dilution, mullet were killed in approximately 60 minutes. A 10 per cent dilution proved toxic in 4 to 8 hours and sometimes not at all. At concentrations below 10 per cent and in controls, mullet were still alive after 72 hours at which time the experiment was discontinued. Death times at specific concentrations increased with decreasing dilutions of the original preparation as indicated by the heights of the vertical lines in figure 1. Thus, at concentrations representing 100, 75, 50, 40, 30, and 20 per cent of the cell-free preparation, the death range for different mullet was 2, 4, 7, 8, 15, and 25 minutes, respectively. At concentrations representing 10 per cent of the original preparation which at times would kill mullet within 4 to 8 hours or not at all, the variability in death time was too great for inclusion in the standard curve.

Comparison of Abbott and Ballantine's (1957) data with ours suggests a similarity of action between the *G. veneficum* and *G. breve* toxins. They reported that a strong toxic culture of *G. veneficum* killed gobies in 5 to 6 minutes. Our usual cell-free preparations killed mullet within 2 to 4 minutes. Preparations diluted to 60 per cent killed within 5 to 6 minutes. Occasionally concentrated preparations were obtained which killed within 30 to 60 seconds.

The toxin unit. One *G. breve* toxin unit (TU) is defined arbitrarily as the amount of toxin per 100 ml. of sample which under standard conditions will kill *Mugel cephalus* in 60 $\pm$ 15 minutes and a two-fold dilution of which may kill within 4 to 8 hours or not at all. The standard conditions include: mullet 2.5 to 3.0 cm. in total length, 100 ml. of test solution contained in 250 ml. beakers, controlled temperature 25 $\pm$ 2° C., immediate use of test solutions (some activity is lost on standing for prolonged periods), and appropriate salinity and pH controls.

Effect of temperature on toxicity. In preliminary experiments, aliquots of living cultures of *G. breve* exposed to lethal temperatures below 10° C. or above 37° C. proved more toxic than the original culture. Freezing-thawing or a 1 to 3 minute exposure at 50° C. were most effective in this respect.

Unialgal mass cultures of *G. breve* and uninoculated control medium were divided into liter aliquots and frozen overnight. After thawing in a water bath at 25° C., aliquots were assayed

and contained 16 TU (killed mullet in 2 to 4 minutes). Other aliquots were filtered through AA millipore filters. Residues (cell debris) were reconstituted with control medium to concentrations representing 20 x the original culture. Little or no activity was detected. The supernate or cell-free preparation contained most of the original activity (16 TU).

The effect of temperature on cell-free preparations containing 16 TU was tested in two series of experiments. In the first, a 2 liter aliquot was placed into a 4 liter flask and heated on a hot plate. Aliquots of 100 ml. were removed at temperatures of 25, 50, 65, 80, and 100° C., cooled immediately to 25° C. in an ice bath, and assayed. Little or no activity was lost from a cell-free preparation heated to 80° C. (Table 1). Approximately 1 TU

TABLE 1

Effect of increasing temperatures on the toxicity of cell-free preparations of Gymnodinium breve *with mullet the assay organism*

Temp. ° C.	Death Time Range (min.)
25	3–4
50	3–4
65	4–7
80	3–8
100	47–80

remained in aliquots of cultures heated to 100° C. In the second series, 2 liter aliquots of a cell-free preparation (16 TU) were placed in constant temperature baths at 25, 45, 55, and 100° C. After reaching the indicated temperatures, 100 ml. aliquots were removed at various times (Table 2), cooled to 25° C. in an ice bath, and assayed. Only 1 to 2 TU could be detected in those samples heated to 45° C. for about 4 hours or to 55° C. for 2 hours. No toxicity could be detected in samples held at 100° C. for 5 minutes.

Effect of pH on toxicity. The influence of pH on the toxicity of a living *G. breve* culture and on the toxicity of a cell-free preparation was tested. Aliquots of a culture (5 weeks old, final pH 8.1, salinity ca. 3.0 per cent, living cell count 1.2 million per liter) and uninoculated control medium were adjusted with 5 N HCl or 5 N NaOH to pH 3.3, 5.4, 7.0, 8.1, and 9.5 (precipitates occurred at the latter pH). At pH 3.3, 5.4, and 9.5, *G. breve* cells were destroyed and mullet were distressed in 17 to 19 minutes. At the original pH 8.1, *G. breve* remained active for an extended period

TABLE 2

Effect of exposure time at elevated temperatures on the toxicity of cell-free preparations of Gymnodinium breve *with mullet the assay organism*

Time (min.)	Average Death Time (min.) 25° C.	45° C.	55° C.	100° C.
0	3.5	3.5	3.5	64
5	..	..	3.5	still alive 72 hr.
15	..	..	4.0	still alive 72 hr.
25	..	3.5	..	still alive 72 hr.
30	3.5	..	15	.....
40	..	..	22	still alive 72 hr.
60	3.5	..	..	.....
65	..	3.5	..	.....
90	..	..	36	.....
120	4.5	..	..	.....
137	..	..	190	.....
150	..	42	..	.....
180	..	..	250	.....
240	4.0	..	...	.....
285	..	120	...	.....

and mullet were distressed in 45 minutes. All fish in pH control vessels appeared normal after 2 hours except for those held at pH 3.3 which showed signs of distress.

A similar test was made with a cell-free preparation containing 4 TU (killed mullet in 5 to 10 minutes). Aliquots were adjusted to pH 5, 6, 7, 8, and 9. The average death times for mullet in solutions at these pH levels were 5, 4, 8, 9, and 7 minutes, respectively. Other aliquots were adjusted to the same pH levels, readjusted to pH 8.0, and then tested. There was no apparent change in toxicity of the original preparation due to pH changes. Controls were still alive after 24 hours.

Within the range of values tested, pH does not modify the activity of the toxin as it affects mullet. However, a radical pH change which destroys *G. breve* enhances the toxicity of the original culture.

The effect of heavy metals on toxicity. The effect of heavy metals on the toxicity of a living culture of *G. breve* was tested. Salts of Sr, Zr, Hg, Ag, and Cu (final concentrations 5 ppm.) were added to 100 ml. aliquots of a culture. Distress times of the guppy and the state of activity of *G. breve* were recorded (Table 3). Hg, Ag, and Cu were lethal to *G. breve* within 7 minutes and guppies showed signs of distress within 13 to 18 minutes. Zr and Sr were less effective in this respect; *G. breve* remained active for ca. 60 minutes and guppies showed signs of distress within 111

TABLE 3

Effect of heavy metals on Gymnodinium breve *and on the toxicity of a living culture with guppy the assay organism*

Metal (5 ppm.)	State of *G. breve*	Average Distress Time for Guppy
Control, no addition	dying—100 min.	120 min.
Sr, as $SrCl_2 \cdot 6H_2O$	dying— 60 min.	183 min.
Zr, as $ZrOCl_2 \cdot 8H_2O$	dying— 60 min.	111 min.
Hg, as $HgCl_2$	dead — 7 min.	18 min.
Ag, as $AgNO_3$	dead — 7 min.	13 min.
Cu, as $CuSo_4 \cdot 5H_2O$	dead — 7 min.	13 min.

to 183 minutes. In controls containing no added metals, guppies were distressed in 120 minutes at which time the numbers of living *G. breve* had decreased considerably. These results indicated that metals which destroy *G. breve* effectively enhance the toxicity of the culture.

Attempts to concentrate the toxin. These experiments indicated that the toxin is confined mainly within the living organism. Attempts were then made to harvest intact cells for possible concentration and isolation of the toxin. Vacuum filtration and slow centrifugation were ineffective because the fragile *G. breve* was destroyed with release of the toxin. Intact cells were absorbed on filter paper but the activities of the reconstituted residues decreased with increasing volumes of the original culture. Thus, the filtrate of several liters of culture contained most of the original activity. However, the residue from smaller volumes (50 to 200 ml.) was absorbed on the filter and the toxin could be eluted from it with little loss of the original activity.

Charcoal and alpha cellulose at various acid and alkaline pH's destroyed or removed the toxin from living cultures and cell-free preparations. Attempts to elute the toxin with organic solvents and buffers failed. Aliquots of a living *G. breve* culture in cellulose dialyzing tubing were dialyzed against several changes of distilled water. Samples removed from the bag after 6 hours (*G. breve* was destroyed) contained most of the original activity.

Summary

Some properties of a toxin in unialgal mass cultures of *Gymnodinium breve* are described. Mullet and guppies were used to compare the potency of different toxin preparations. *G. breve* is extremely sensitive to chemical and physical manipulation

and the toxicity of a living culture is increased by agents which destroy *G. breve*. Such observations indicate that the toxin is largely within the cell. However, the fragility of *G. breve* has rendered fruitless attempts to concentrate intact cells as a means of concentrating the toxin.

In the absence of further information, it is difficult to compare the toxin of *Gymnodinium veneficum* (Abbott and Ballantine, 1957) with the toxin of *G. breve*. They are probably similar. Although the *G. veneficum* toxin has been described as an exotoxin (secreted into the water), it is difficult to tell whether *G. breve* produces a true exotoxin (freely secreted or excreted into the environment by living cells) or an endotoxin (liberated from the cells by lysis or autolysis).

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STUDIES ON THE ABSORPTION OF HYDROCORTISONE FROM THE COLON OF PATIENTS WITH IDIOPATHIC ULCERATIVE COLITIS[a]

MARCEL PATTERSON

The use of corticosteroids topically in the treatment of chronic ulcerative colitis was first described by Allodi and Muratori (1956). More extensive therapeutic trials have been reported by Truelove of Oxford University (1956, 1957). The latter observed remissions in about two-thirds of cases so treated. More recently other reports have appeared (2, 4, 8).

Our experience with 21 patients has been reported in detail elsewhere (6). Most of these cases were early and relatively mild ulcerative colitis involving the rectum and sigmoid. The treatment consisted of instillations four times a day of a rectal suppository containing 10mgm. of Hydrocortisone acetate (Wyanoids HC, Wyeth) or an instillation twice daily of a suspension of 50 mgm. of Hydrocortisone acetate in water. In 78 per cent of cases a rapid remission was obtained.

By using a radiopaque proctoclysis, we have observed that solutions could be introduced by retrograde flow into the cecum and terminal ileum. This has encouraged us to attempt therapy of more extensively diseased colons. In four patients treated with water soluble hydrocortisone (Solu-Cortef, Upjohn) 100 to 200 mgm. dosage in 500 cc. of normal saline, a rapid remission was observed in two. It is recognized that this group is too small to evaluate the place of this method of therapy in this kind of patient but the results were most encouraging except where far advanced tissue changes had occurred.

The distinct advantage of this form of therapy in our experience was in the speed in which remissions occurred. Often a striking improvement in the appearance of the bowel could be observed by proctoscopy within 2 to 3 days and by the end of two weeks the mucosa appeared healed.

Theoretically, there should be maximum concentration of the medication in the area treated. But the question immediately arose whether the effects observed were the results of systemic

absorption or a topical one. This question cannot be answered absolutely although in our experience and that of others (11) mucosal changes do not disappear with great rapidity following systemic corticosteroid therapy. Aside from clinical observations, the measurement of plasma levels of hydrocortisone and of urinary metabolites of hydrocortisone might be of help in estimating the systemic effect. With this as an object, this study was undertaken in five patients with chronic idiopathic ulcerative colitis.

Method

In three patients, plasma hydrocortisone levels were measured, by the method of Silber and Busch (9), before and at hourly intervals for four hours after the insertion of a rectal suppository containing 10 mgm. of hydrocortisone acetate (Wyanoids HC). A fourth patient was studied in a similar manner after the rectal instillation of 50 mgm. of hydrocortisone sodium succinate (Solu-Cortef, Upjohn) in normal saline.

Urinary 17 ketosteroids were measured by the method of Zimmermann *et al.* (12) and 17 hydroxycorticoids were measured by the method of Reedy *et al.* (7). Twenty-four hour urine samples were obtained on the day before and the day of the administration of 50 mgm. of hydrocortisone sodium succinate in 250 cc. of normal saline given as a proctoclysis. These studies were done on two patients with idiopathic ulcerative colitis involving the entire colon. One of these patients is the same patient (IV) in which hourly blood levels were taken.

Results

The following table depicts the plasma levels of hydrocortisone observed:

Plasma Levels of Hydrocortisone μg Percent Noted Following a 10 mgm. Rectal Suppository of Hydrocortisone Acetate (Wyanoid, HC):

	control	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
Patient I	15	16.6	15.8	12.5	21.9	21.5
Patient II	17.5	28.2	31.0	...	30.0	...
Patient III	15.8	13.8	13.7	14.7	16.0	...
Following 50 mgm. of Hydrocortisone Sodium Succinate in 250 ml. of saline as a proctoclysis. (Solu-Cortef)						
Patient IV	16.7	16.7	16.3	12.9	...	11.2

The following table indicates the urinary metabolites measured in 24 hour urine collections following a proctoclysis of 50 mgm. of hydrocortisone sodium succinate in 250 ml. of normal saline.

	Creatanine Gm/24 hrs.			17 Ketosteroids μg%/24			17 Hydroxy-corticoids μg%		
	Control Day	Rx Day	Rx+1 Day	Control Day	Rx Day	Rx+1 Day	Control Day	Rx Day	Rx+1 Day
Patient IV	.77	.41	.42	11.01	4.27	4.62	6.22	9.06	4.84
Patient V	.68	.70	..	17.76	15.83	...	5.02	5.22	...

Discussion

Absorptive studies by Liddle (3) indicated that there was 26 per cent absorption (oral absorption considered 100 per cent) from the normal rectum following the administration of 40 mgm. suppositories of delta, 9-fluorohydrocortisone. No studies were done in patients with diseased colons.

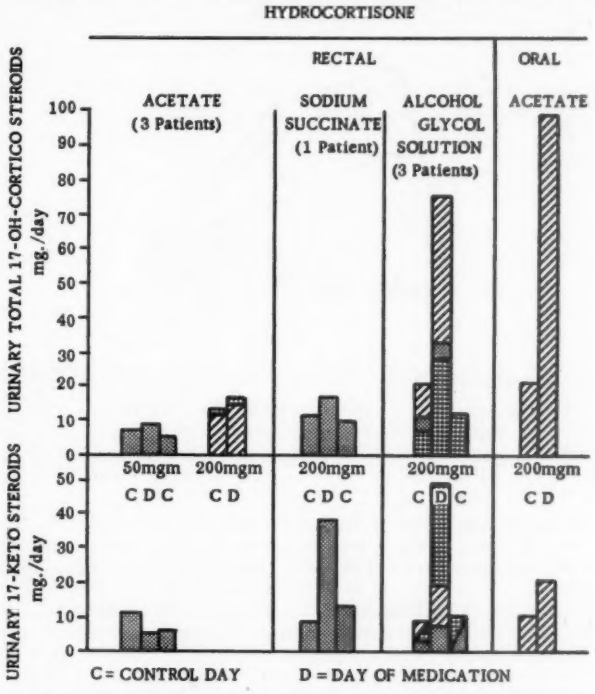
Schwartz *et al.* (8) found only minimal rises in plasma levels of hydrocortisone following 200 mgm. hydrocortisone sodium succinate retention enemas in patients with ulcerative colitis. Also, they noted comparable low increases in plasma radioactivity after giving radioactive labeled hydrocortisone retention enemas at similar dosage levels.

Navarro *et al.* (4) measured 24 hour urinary metabolites of hydrocortisone to detect colonic absorption in patients with ulcerative colitis. They noted a considerable variation in the amount of metabolites excreted when the form in which the material was administered was varied. For example, there was no increase in urine corticosteroid metabolites following rectal instillation of 200 mgms. of hydrocortisone acetate; only slight increase following an aqueous solution of 200 mgm. hydrocortisone sodium succinate and a considerable increase occurred when hydrocortisone free alcohol was given as a suspension or a suppository.. (See Fig. 1.)

Our findings in this small group of studies are similar to those described by Schwartz *et al.* (8) and Navarro *et al.* (4). They suggest that there is very minimal absorption of hydrocortisone acetate and hydrocortisone sodium succinate from the colonic mucosa involved with ulcerative colitis.

These findings of poor absorption are more striking when compared with the effects noted following the administration of oral

URINARY EXCRETION OF HYDROCORTISONE AND METABOLITES AFTER VARIOUS ROUTES OF ADMINISTRATION IN ULCERATIVE COLITIS PATIENTS

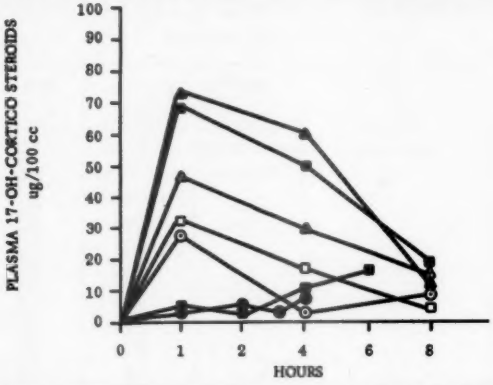


Data for 200mgm. dosage from studies by Nabarro, J. D. N.; A. Moxham; G. Walker; and Slater, J. D. N. -Brit. M. J. II: 272, Aug. 3, 1957.

FIGURE 1

hydrocortisone. (See Fig. 2.) Here, there is prompt rise in plasma levels of hydrocortisone, *i.e.* in a study by Nelson *et al.* (5) the plasma level of hydrocortisone increased 78 ug per cent (range 45–116) in one hour and at four hours dropped to 43 ug per cent (range 11–52) following the oral administration of 200 mgm. of hydrocortisone acetate. Also, these authors reported in normal persons a diurnal variation in plasma hydrocortisone levels up to 9 ug per cent. This observation must be considered in interpreting any changes observed in plasma measurements.

COMPARISON OF PLASMA LEVELS OF 17-OH CORTICOSTEROIDS FOLLOWING ORAL AND RECTAL INSTILLATION



MEAN OF DIFFERENCE FROM ZERO TIME CONCENTRATION

⊖ = 12.5 mgm.
□ = 25 mgm.
△ = 50 mgm.
■ = 100 mgm.
▲ = 200 mgm.

ORAL HYDROCORTISONE ACETATE

Studies from D. H. Nelson; A. A. Sandberg; J. G. Palmer; and F. H. Tyler. J. of Clin. Invest. - 31:843, 1952.

HYDROCORTISONE ACETATE RECTAL SUPPOSITORY (3 Patients)

● = 10 mgm.
■ = 200 mgm.

Studies from John Sealy Hosp. Exp. Med. Lab.; U. of Tex.

HYDROCORTISONE SODIUM SUCCINATE RECTAL ENEMA (5 Patients)

Studies from R. D. Schwartz; G. L. Cohn; P. K. Bondy; G. V. Upton; and H. M. Spiro. Proc. of Soc. Exper. Biol. and Med. In press.

FIGURE 2

This relative lack of absorption suggests that the effect on the colonic mucosa following the topical administration of hydrocortisone is a local one rather than systemic and is comparable to that seen in topical steroid therapy of skin, eye or joint inflammations.

Another distinct benefit of topical treatment, aside from the advantage of inducing rapid remissions, is the lack of unpleasant side effects which are often noted following prolonged systemic steroid therapy. We believe this lack of side effects is related to the poor absorption.

The follow-up interval on our group of patients is too short to predict with certainty the duration of these induced remissions. So far, relapses have occurred in two patients, one occurred two

years and one 16 months following treatment. In both instances, a favorable response was obtained to a second course of therapy.

Summary

Plasma hydrocortisone levels measured in three patients with ulcerative colitis after the rectal instillations of hydrocortisone acetate in 10 mgm. suppositories and in one patient following a proctoclysis of 50 mgm. hydrocortisone sodium succinate in 250 cc. of normal saline indicated only slight changes.

Nor were significant increases noted in the 24 hour urinary excretion of 17 ketosteroids and 17 corticosteroids in two patients with ulcerative colitis following the rectal instillation of 50 mgm. of hydrocortisone sodium succinate in 250 ml. of normal saline.

These findings are further evidence that the observed salubrious effects of topical steroid therapy in ulcerative colitis are a local one. The advantages of minimal absorption are: fewer side effects and the possibility of using steroids in situations where this therapy might otherwise be contraindicated, *i.e.* ulcerative colitis and tuberculosis or peptic ulcer.

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[b] With the technical assistance of Alene Bennett and Elizabeth Heidrick.

CALLING ATTENTION TO

SEPTEMBER 1958

MEDICAL IMPRESSIONS OF JAPAN: Underlying economic anxiety met by cheerful industriousness; no tendency toward drug addiction although alcohol consumption is increasing; broad health consciousness among people with sophisticated self-medication, & good governmental control over well labelled drugs; excellent drug manufacturing (Takeda Pharmaceutical Industries, Osaka, has plant equalling any American one, with superb library and new labs); 50 medical schools (classes from 50 to 80 students each of 4 years) changing from conventional German pattern to more fluid American program; labs need equipment, libraries need journals, have keen competition among keen students; physicians consulted chiefly in crises, when hospitals are used; teaching tospitals & clinics improving; residency training soon to be undertaken; population control on way; in face of modern ugliness the ancient beauties are revered, & people preserve self-esteem & sense of responsibility.

JAPANESE ITEMS: Great interest among microbiologists in antibiotics; Japanese Antibiotic Research Association publishes *Journal of Antibiotics* now in 10th year, with many important reports on encephalomycin, kanomycin, leucomycin, mitomycin, sarkomycin, & trichomycin. Y Kobayachi & Co continue studies on antidiabetic action of mesoxalates (*Jap J. Pharmacol* 1/9/51; 4/109/55; *Proc Jap Acad* 34/387/58). B Nuki studies pharmacology of pulmonary & coronary circulation (*Jap Circul J* 18/No 4/July 54; 21/ No 6/ Sept. 57). K Shimamoto & Co find curariform hypotensive ganglionic blocking action of menisperine, magnaflorine & related alkaloids from cocculus & magnolia (*Jap J Pharmacol* 7/135–156/58). H Takagi & Co note antagonism between LSD & chloropromazine & reserpine (*Jap J Pharmacol* 7/119–134/58).

RUSSIAN ITEMS: IS Beritov describes spatial projection of perceived enviromental objects by means of labyrinthine receptors (*Sechenov Physiol J USSR* 43/561/58). AG Ginetsinski & Co greet Leon A. Orbeli on his 75th birthday (*ibid* p 557). KS Koshtojants discusses site of cardiac action of potassium (*ibid* p 631). AV Palladin & Co study proteins of brain & nerve (*Ibid* p 572). AI Polezhaeva notes effect of diphenylacetic acid ethers on cough reflex (*Pharmakol & Toksi* 20/56/58). NG Poliakov notes structure-action relations in phenylethanolamines (*ibid* p 21). JI Rautenstein describes development of Russian microbiology (*Microbiol* 26/625/58). E. Simonson reviews recent Russian reports in cardiovascular physiology (*Ann Rev Physiol* 20/123/58). SI Zolotukhin reviews penicillin on blood coagulation (*Pharmakol & Toksi* 20/80/58).

HISTORICAL: BI Cohen edits *Isaac's Newton's Papers & Letters on Natural Philosophy* (Harvard Univ Press, Cambridge, Mass, 58 501pp $12.5). TH Grainger offers *A. Guide to the History of Bacteriology* (Ronald Press, NY, 58, 176pp, $4.5). ES Lowman describes surprising adventure in red-tape in

re her report on USSR education (*Sat Rev* Aug 9/58 p7). HR O'Brien notes dawn of Medical education in tropical Africa (*J Med Educ* 33/559/58). Lord Russell of Liverpool documents Japanese war atrocities in *The Knights of Bushido* (Dutton, NY, 58, 334pp, $5). KT Steinitz gives superb bibliography of Leonardo da Vinci's *Trattato della Pittura* (Munksgaard Copenhagen, 58, 244pp, $7). RO Steuer & JBdeCM Saunders issue important *Ancient Egyptian & Cnidian Medicine: The Relationship of the Aetiological Concepts of Disease*, showing transfer of Egyptian medical ideas to ancient Greece (University of California Press, Berkeley, 58, 96pp, $3). H Schuman well describes history of medicine collection developed at Duke Univ by Josiah C. Trent 1914–1948 (*Bull Med Lib Asso* 46/352/58). I Welt discusses indexing of drug action (*ibid* p 367).

CLINICAL: M Burnet notes growing leukemia epidemic (*Sat Rev* Aug 2 p 38). A Feldstein & Co find no causal relation between serotonin & Schizophrenia (*Science* 128/358/Aug 15/58). RC Merrill reviews estriol (*Physiol Rev* 38/463/58). Our L Sapirstein & Co well study regional blood flow by radioactive indicators (*Am J Physiol* 193/161, 272/58; *J Appl Physiol* 13/81/58). WJ Schull & JV Neel find increased male sex ratio in children of A-bomb survivors suggesting induced sex-linked lethal mutations (*Science* 128/343/Aug 15/58). SJ Segal & WO Nelson come with another orally active antifertility agent, diethylamino-ethoxyphenyl-phenyl-p-anisylethanol (*Proc Soc Exp Biol Med* 98/431/58). WG Shafer & Co find Na-diphenylhydantoinate increase tensile strength of healing wounds (*ibid* p 348). SG Tuttle & MI Grossman suggest reflex of gastric contents is factor in esophagitis (*ibid* p 225). RO Wallerstein & SR Mettier edit *Iron in Clinical Medicine* (University of California Press, Berekeley, 58, 304pp, $6). EG Yonehiro & Co suggest oral use of radioactive iron to detect occult blood in stools (*Proc Soc Exp Biol Med* 98/339/58).

C. D. LEAKE

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